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Application of the otolith weight – age relationship for the pilchard, *Sardinops sagax neopilchardus*

W.J. Fletcher

Abstract: Adult pilchard (*Sardinops sagax neopilchardus*) from southwestern Australia held in fish cages for 1 year showed minimal increase in mean length but a steady increase in mean otolith weight (1.61–1.83 mg); this increase (0.22 mg) closely parallels the previously calculated mean difference between adjacent age classes (0.24 mg). Modes in otolith weight frequency distributions of fishery-caught pilchards, previously suggested as corresponding with separate year classes, generally persisted and progressed through subsequent samples. By contrast, fish length frequency distributions of these samples showed no consistent modal progression. The average weight of otoliths for year classes 2–8 were used to calculate growth rates: following sexual maturity, at age 2, males grew to a significantly smaller maximum size than females (L_∞ = 162 and 172 mm respectively). The otolith weight – age relationship, validated by following a cohort of unusually low abundance as it moved through the fishery, indicated that recruitment to the fishery generally began at age 2 but was not complete until age 4. These data have been successfully used in forecasting the catch rate of the fishery up to 2 years in advance and providing an estimate of the rate for natural mortality of 0.43.

Résumé : Des sardines australiennes adultes (*Sardinops sagax neopilchardus*) provenant du sud-ouest de l'Australie, maintenues en cage pendant 1 an, n'ont montré qu'un faible accroissement de la longueur moyenne des otolithes, mais une augmentation constante du poids moyen des otolithes (de 1,61 à 1,83 mg). Cette augmentation (0,22 mg) correspond de près à la différence moyenne entre les classes d'âge adjacentes calculée antérieurement (0,24 mg). Les modes observés dans les distributions de fréquence du poids des otolithes des sardines australiennes dans les prises commerciales, antérieurement attribués à des différences de classes d'âge, ont généralement persisté ou progressé dans les échantillons subséquents. Par contre, les distributions de fréquence de la longueur des poissons de ces échantillons n'ont pas révélé de progression modale cohérente. Le poids moyen des otolithes des classes d'âge 2–8 a servi à calculer les taux de croissance : une fois devenus sexuellement matures, à 2 ans, les mâles atteignent une taille maximale significativement plus petite que les femelles (L_∞ = 162 et 172 mm respectivement). Le rapport entre le poids des otolithes et l'âge, validé par l'étude d'une cohorte d'abondance inhabituellement faible à mesure qu'elle se manifestait dans les prises, a indiqué que le recrutement dans la pêche commence généralement à l'âge de 2 ans, mais qu'il n'est pas complet avant l'âge de 4 ans. Ces données ont été utilisées avec succès pour prévoir le taux de prises jusqu'à 2 années à l'avance et pour calculer un taux estimatif de la mortalité naturelle de 0,43.

[Traduit par la Rédaction]

Introduction

The concept of using otolith weight to estimate the age of fish is not new, having been suggested nearly 40 years ago by Templeman and Squires (1956). Despite promise this method has, nevertheless, been largely ignored as a potential method of ageing (Brander 1974; Boehlert 1985). The realisation that the growth of otoliths is partially decoupled from the increase in the somatic size of fish has now been well documented (Spratt 1972; Mosegaard et al. 1988; Reznick et al. 1989; Molony and Choat 1990; Wright et al. 1990). Such data have, therefore, provided further support that otolith weight may provide a practical method for estimating the age of individuals.

Direct testing of the relationship between otolith weight and age has been relatively rare. Boehlert (1985) found that a combination of otolith weight and other objectively measured variables, such as fish length, had a similar precision for ageing *Sebastes diploproa* individuals to that

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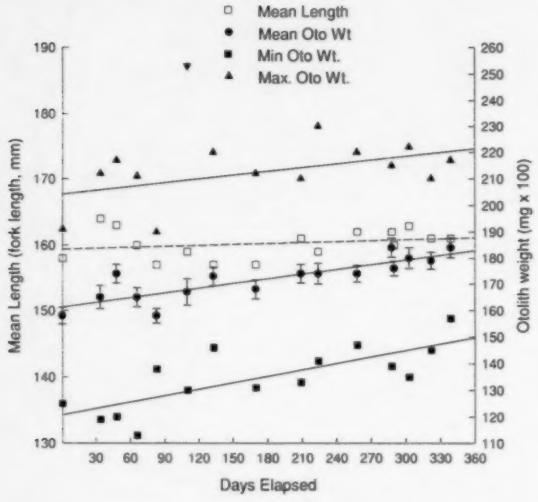
Fig. 1. The mean fish length (fork length), mean otolith weight (mg, ×100), and the smallest and largest otolith weight (mg, ×100) of the samples of pilchards taken from the captive fish maintained in the fish cages. Note that one outlier for the largest otolith in the sample was omitted from the analysis owing to its unrepresentative influence on the regression.



obtained by reading annuli. Similarly, the ages of over 90% of *Sardinella aurita* (Pawson 1990), *Nototheniops mudifrons* (Radtke and Hourigan 1990), and Pacific marlin (*Makaira nigricans*; Wilson et al. 1991) have been correctly estimated using combinations of otolith weight and otolith length. There are, however, no reported studies that have used otolith weight as a method of ageing to provide long-term catch-at-age data to monitor a fishery.

In the precursor to the present study, Fletcher (1991) showed that despite a large overlap in the lengths of individuals of adjacent ages, there was little overlap of otolith weights among a number of age classes for the pilcard *Sardinops sagax neopilchardus*. Of greater interest was that there were modes in a frequency distribution of otolith weights that corresponded closely to the mean weights of each of the previously identified age classes. These results were, therefore, consistent with otolith weight being a reliable estimator of age, but further work was required to confirm this hypothesis. It was important to investigate whether these modes in the otolith weight frequency distribution persisted and progressed through time. Moreover, it was essential to demonstrate that any ages ascribed using this method could provide useful catch-at-age data to monitor the local pilchard fishery.

The present study followed, in detail, changes in the otolith weight distribution of pilchards over a period of 1 year for a captive population and 2 years for wild-caught fish. These data were used to determine whether the relationship between otolith weight and age reported by Fletcher (1991) was maintained. Subsequently, the relationship between otolith weight and age was applied to the longer term (5 years) catch data for the Albany fishery.

Materials and methods

Captive fish

In October 1991, 1.5 t of pilchards (approximately 40 000 individuals) were caught by a professional purse-seine fisherman in Cockburn Sound (116.5°E 32°S) and towed 4 km to Fremantle harbour. From the bunt of their net, approximately half the fish were released in a fish cage (3.5 × 2.5 × 4 m) and some of these were subsequently moved to a second cage a few days later. The fish were fed three times a week and any fish that died were removed and recorded until the experiment ended in September 1992. Initially, the fish were fed with pellets designed to feed chickens but this was later changed to trout starter pellets. Samples of 25 fish were taken at approximately monthly intervals, with the length (fork length) and weight of each individual measured and their otoliths (sagittae) removed. The otoliths were washed in distilled water, dried to a constant weight in an oven at 60°C for at least 12 h, and then weighed to the nearest 0.01 mg.

Wild-caught fish

Random samples of pilchards captured by a purse seine fleet operating in the Albany region of Western Australia (118°E, 35°S) were obtained every month from January 1989 to December 1993. In each month, the lengths and weights of approximately 300–500 individuals were measured from a total of 15–20 separate catches. Otoliths were removed from 100 randomly selected individuals from each of these samples and weighed as above. Every third month from September 1989 to September 1991 a larger sample of otoliths was obtained from the remaining 200–400 individuals in the monthly sample.

The resultant distributions of otolith weights in each of the months where large samples of otoliths were obtained were examined to determine the presence, number, and location of modes. This examination was done using MIX (MacDonald and Green 1988) to determine the position of the modes in each month.

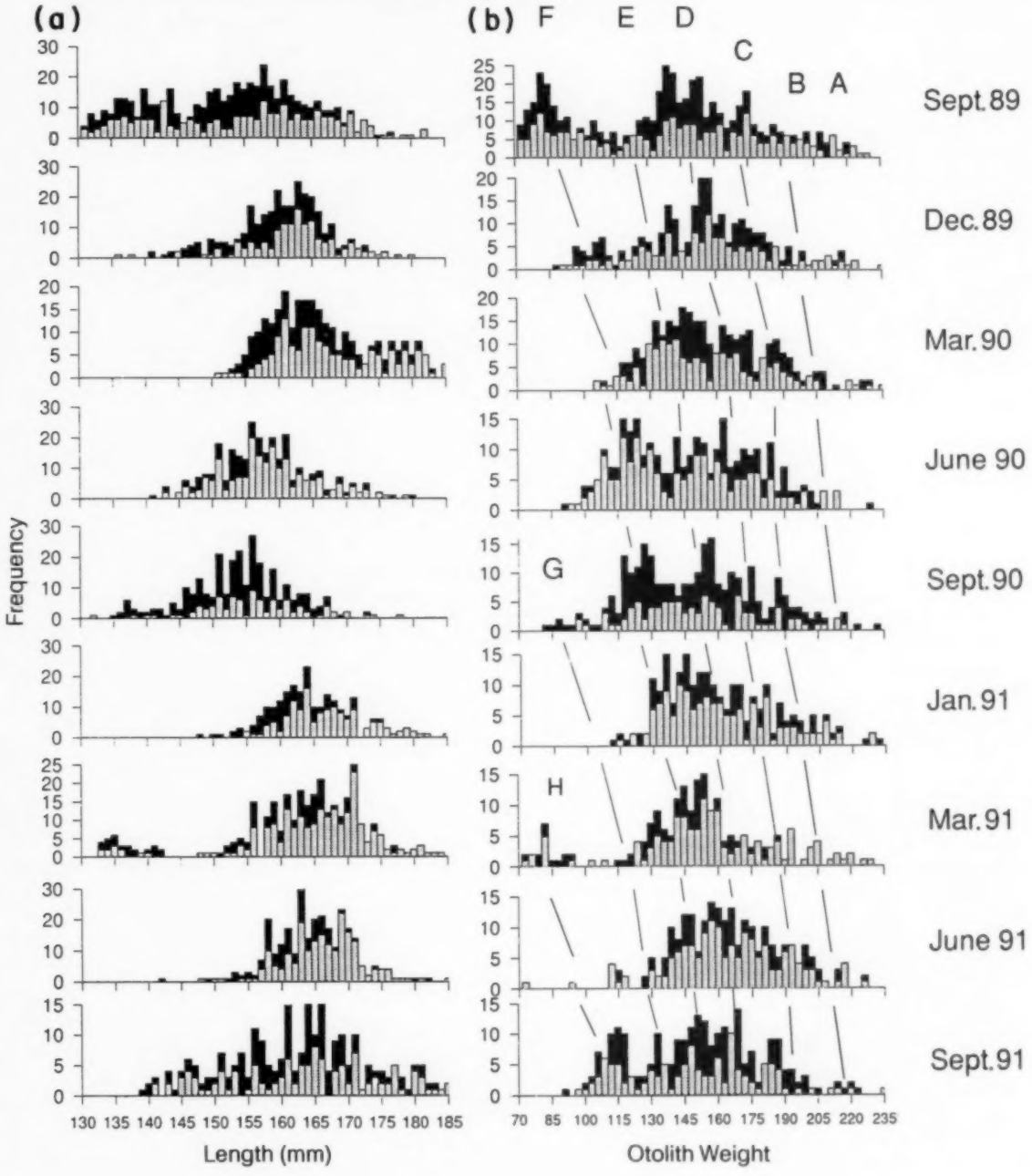
The frequency distributions of otolith weights for each month from 1989 to 1993 were weighted by the estimated number of pilchards caught in that month, this being estimated by dividing the weight of pilchards caught by the fishery with the average weight of individuals caught. These weighted monthly distributions were then summed over each of the years to create the annual otolith weight catch curves.

Results

Captive fish

The pilchards survived capture and the transfer to the cages with minimal mortality, especially after the first 2 weeks. Unfortunately, significant theft of fish occurred during the initial period which precluded any analysis of total mortality. The mean lengths of the fish from the cages did not increase greatly during their year in captivity, the initial mean length was 158 mm and the final value was just 161 mm (Fig. 1). By contrast, the average otolith weight of the samples, along with the weight of the smallest and largest otolith found in each of the samples, increased steadily throughout the year (Fig. 1).

Fig. 2. The length (*a*) and otolith weight (*b*) frequency distributions for the period September 1989 to September 1991. The lines on (*b*) connect cohorts as determined by MIX. The distributions for females are indicated by the grey regions; the values for males and females combined are indicated by the black regions.



A regression analysis indicated that the mean otolith weight increased from 1.61 mg in October 1991 to 1.83 mg over the 12-month period with a correlation coefficient $r = 0.85$ ($n = 16$, $P < 0.01$). The average annual increase for all captive pilchards was 0.22 mg. The data for the smallest and largest otolith weight in each sample was more variable with the correlation coefficients for the regressions being $r = 0.76$ ($n = 16$, $P < 0.01$), $r = 0.54$ ($n = 15$, $P < 0.05$),

Table 1. Mean otolith weights (mg) for cohorts in September of 1989–1991.

Age-class	September 1989 (Fletcher 1991)	September 1989 (recalculated)	September 1990	September 1991
2	0.9	0.89	0.9	1.08*
3	1.35	1.32	1.26	1.30
4	1.52	1.50	1.52	1.47
5	1.76	1.72	1.70	1.66
6	2.06	1.93	1.92	1.89
7		2.19	2.18	2.15

*For the age 2 data in 1991 it should be noted that recruitment first occurred in March not September as in 1989 and 1990.

Table 2. Mean otolith weights and the calculated separation values between adjacent age classes of pilchards in the Albany region.

Age	Mean otolith weight (mg)	Upper separation value (mg)
2	96	114
3	125	138
4	150	163
5	174	185
6	195	205
7	215	225
8	235	

respectively. The average increase in weight over the year for the smallest otolith was slightly greater than the mean at 0.28 mg and slightly less for the largest otolith at 0.18 mg.

Persistence and progression of modes (wild-caught fish)

Length–frequency distributions

There was little pattern in the length–frequency distributions of pilchards obtained from the Albany fishery over 2 years (Fig. 2*a*). The number of visible modes was generally only one or two. Furthermore, the range in the distribution of lengths varied erratically among sampling periods showing no obvious progression through time from which growth rates could be calculated. This situation was improved only slightly when just females were plotted (Fig. 2*a*).

Otolith weight – frequency distributions

The modal structure present in the otolith weight distribution from the September 1989 sample, has already been shown to correspond with age-classes determined from an examination of annuli on the otoliths (Fletcher 1991). Over the subsequent 2 years of sampling, cohorts could be discerned to varying degrees in each of the distributions (Fig. 2*b*). For example, cohort F, which in September 1989 had a mean value of 0.89 mg, had by September 1991 increased in weight to 1.50 mg. The distinction among the otolith weight modes was often poorly defined because of the bimodal nature of some cohorts. This was frequently related to slight differences between sexes with the distributions for females often showing clearer modes than when both sexes were combined (e.g., March and June in 1990 and 1991). Despite these problems, the cohorts were sufficiently distinct in most months to allow their discrimination and progression to be monitored. The increases in the mean weight of the cohorts as calculated by MIX were examined against the predicted increases determined from the original annuli method of ageing (Fletcher 1991; Table 1).

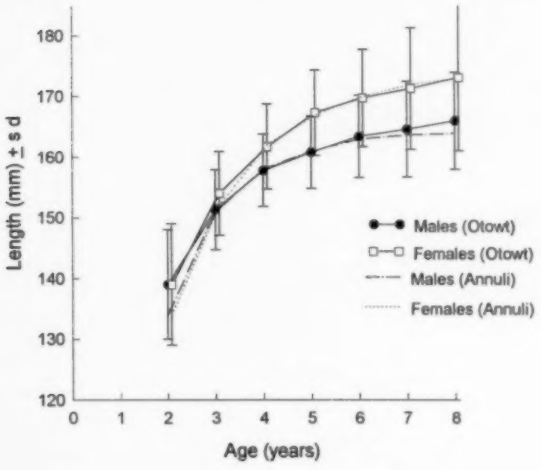
In nearly all cases, the yearly increase in the otolith weight of modes was similar to that predicted. There was a difference in the increase in otolith weight among age classes; age 2 individuals increased by an average of 0.38 mg in a year but the older groups increased by only 0.21 mg, with the mean increase for all groups being 0.24 mg. There was some variation in the exact position of the modes among years, but this was generally less than 0.05 mg with a tendency for the discrepancy to increase with age. The difference between the observed and expected positions for the age 6 modes was largely due to an error in the original study. Fletcher (1991) had (owing to limited data) incorrectly assumed that age 6 was the oldest age class in this population. Following the cage studies, further analysis of modal progressions and the examination of growth zones from 1500 individuals (W.J. Fletcher, unpublished), ages up to 8 years old have been assumed in this study.

There was some variation in the timing of recruitment to the fishery among the 3 years. Thus, otoliths in the 0.8- to 0.9-mg range were found in September of 1989 and 1990, whereas in 1991, this size group was present in March. By September 1991, this cohort had a mean weight of 1.08 mg (Table 1). There is, however, evidence of a secondary peak in this region for cohort F in the September 1989 sample and in later samples.

Estimated growth rate

To calculate the growth rate of individuals using otolith weight as an estimate of age it was necessary to determine the values that separated the different age classes. The mean otolith weight of each of the assumed age classes 2–7 in June of each year was used as the reference point and the weight for each age class was then determined by calculating the mean value for the 3 years, with the separation between adjacent classes being defined as midway between the mean values (Table 2). Owing to the deterioration of modal structure in the older age groups, the position of the age 8 individuals was an assumed value using the mean annual increase in otolith weight.

Fig. 3. Fork lengths (mm, mean ± SD) for males and females for each of the ranges in otolith weight corresponding to age-classes 2 to 8. The values for each sex have been offset slightly to improve clarity. Separation values between age-classes are located in Table 2.



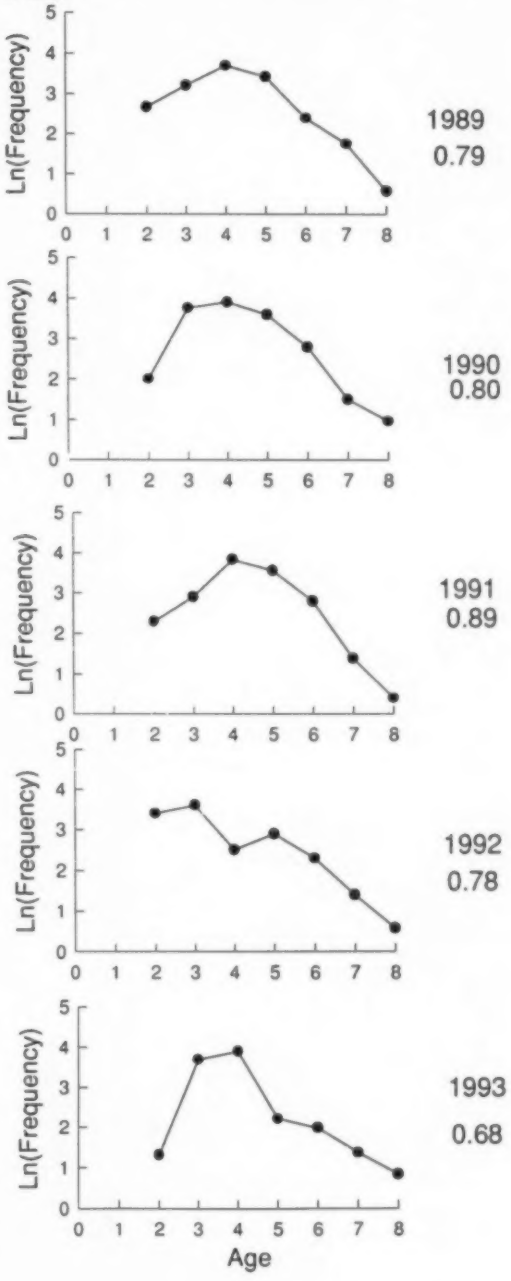
Using these values the average length of males and females with otolith weights in these different ranges were calculated (Fig. 3). There were clear differences between male and female pilchards in the mean lengths for a given age class (otolith weight range). After their second year, when sexual maturity is reached (Fletcher 1990), females grow faster than males attaining much greater lengths ($F_{age \times sex} = 7.35$; df = 8, 5835; $P < 0.001$; Fig. 3). The von Bertalanffy curves fitted to these data indicated that for males $K = 0.728$ and $L_\infty = 164$ mm; whereas for females $K = 0.6629$ and $L_\infty = 172$ mm. These growth curves are very similar to those generated for this population using the annuli method of ageing (Fig. 3) from which similar von Bertalanffy values were calculated with $K = 0.805$, 0.584 and $L_\infty = 164$, 174 mm for males and females, respectively (W.J. Fletcher and S.J. Blight, unpublished data).

Yearly catch curves/mortality estimates

The weighted catch curves for the 5 years (1989–1993) are shown in Fig. 4 with the values for each year class integrated into the number of individuals caught per year and plotted on a logarithmic scale. These indicate that pilchards begin to recruit to the purse-seine fishery off Albany during their second year but were generally not fully recruited until they have reached 4 years, which equates to a size of approximately 160 mm. The weak 2-year-old class that recruited in 1990 (cohort G) can be clearly seen passing through the fishery in this format.

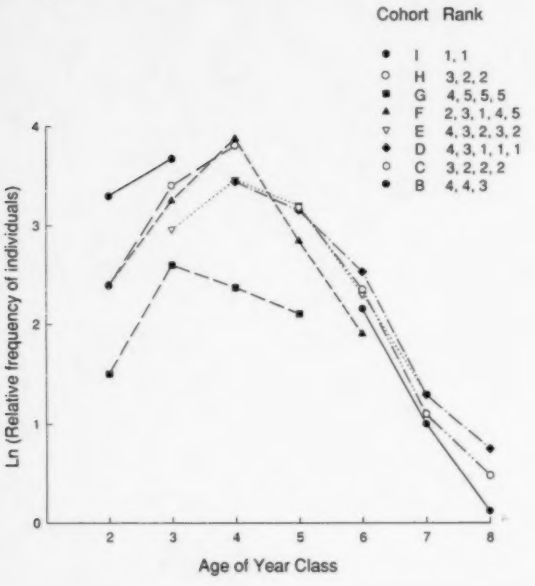
The overall rate of mortality (Z) after age 3 increased for the first 3 years of the study, from 0.79 per year in 1989 to 0.90 in 1991, a period when the level of effort (E) for the fleet was approximately 400 000 L of fuel. Subsequently, Z fell to 0.78 in 1992 and 0.68 in 1993 when the effort had been reduced by 33%, to approximately 300 000 L.

Fig. 4. Catch-at-age curves for the years 1989–1993. Total mortality (Z) was calculated for year-classes 4 to 8 only.



Thus, using these variations in effort with the changes to the average rates of total mortality (by solving simultaneous equations of the relationship $Z = M + qE$) over the two periods suggests that natural mortality (M) for this stock is approximately 0.43.

Fig. 5. The relative number of individuals in each year-class caught by the fishery scaled by the total number of fish caught per year. Letters refer to the cohorts defined in Fig. 2.



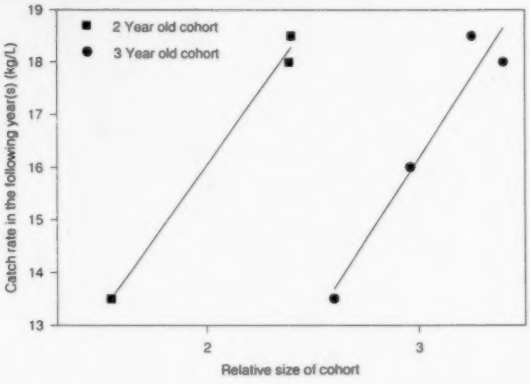
There was a high level of agreement between the variations in the relative size of a cohort caught in one year with the number of the cohort caught in the next, especially up to age 4 (Fig. 5). For example, in both 1989 and 1991, there were greater than average abundances of 2-year-old fish. These were followed by larger than normal amounts of 3-year-old fish in 1990 and 1992, with higher numbers of 4-year-old fish in both 1991 and 1993 (although this did not continue for ages 5 and 6). Conversely, there were relatively few 6-year-old fish in 1989 (cohort B), and a similarly depressed 7-year-old cohort in 1990 and few 8-year-old fish in 1991. Finally, the clearest example was the small number of 2-year-old fish found in 1990, which led to a correspondingly small number of 3 year olds in 1991 (cohort G) and relatively few 4 and 5 year olds in 1992 and 1993, respectively. This had serious implications for the fishery with very poor catch rates experienced by the fleet in 1992. Thus, there appears to be a good relationship between the relative size of a cohort as 2- and 3-year-old fish with the subsequent catch rate of the fishery 2 years and 1 year later (Fig. 6).

The limitation in the otolith weight method ability to estimate age will, however, probably result in a loss of precision in the assignment of some individuals within older age classes with the smearing of a cohort between adjacent ages, especially when there is a large disparity in their relative sizes. This may explain the atypical trajectories of cohorts F and G after age 4 (Fig. 5).

Discussion

The hypothesis that the otolith weight of individual pilchards provides realistic estimates of their age is largely supported

Fig. 6. The catch rate of the fishery (kg of fish caught/L of diesel used) in comparison to the relative size of the 2-year-old cohort 2 years previously and the relative size of the 3-year-old cohort 1 year previously.



by this study. Otolith weights of captured individuals increased despite little growth in their body length, further supporting the generally stronger association between age and otolith weight than with length (Mosegaard et al. 1988; Reznick et al. 1989). Furthermore, the increases in the mean, minimum, and maximum otolith weights of individuals over the year in captivity mirrored the differences found among modal weights of wild-caught fish. Finally, the modes in the otolith weight frequency distributions, whilst not always totally clear, appeared to persist and progress in a fashion which was generally consistent with them representing different year classes.

The double peaks, which appear to be a common trait of the combined otolith weight cohorts (Fig. 2*b*), along with the 6-month difference in the time when recruits were seen among years, are consistent with the pattern of spawning for pilchards in the Albany area. Plankton sampling (Fletcher and Tregonning 1992) and gonad samples (W.J. Fletcher, unpublished) show that there are two distinct spawning periods, one in July and another in December. The relative success of spawning from these two periods is probably responsible for some of the variation in the mean otolith weights of age classes seen among years.

A further cause of bimodality in the older age classes results from slight differences in the modal weights between male and females owing to their different lengths at age. The weights of female cohorts were slightly different from males with this small sexual variation consistent with Pawson (1990) and Beckman et al. (1991) who each found that length along with otolith weight was a significant covariant when estimating age.

There was a large difference in the growth rate between the sexes with females attaining significantly larger lengths than males for the same otolith weight (age). This had not been examined in previous studies on Australian pilchards (e.g., Blackburn 1949; Baker 1972; Joseph 1981). An increased rate of growth for females compared with males, once sexual maturity is reached, is a feature of many species of fish. Specifically, this pattern has been found for the South African pilchard, *Sardinops sagax ocellatus*,

where females grew larger than males, with the differences being more pronounced in older age groups (Baird 1970). The difference in the apparent rate of growth between individuals with similar sized otoliths provides further evidence that the weight increases in otoliths are not wholly coupled with increases in length.

Despite the minor variations in the position of modes among years the relative size of each cohort was generally similar from one year to the next, particularly for age classes 2–4. Therefore, the examination of the yearly catch curves based on the use of otolith weight classes to estimate age has provided useful information for monitoring and stock assessment studies of the Albany fishery. The catch-at-age curves for 1989–1993 have provided the first evaluation of the status of the pilchard stock off Albany. The natural mortality of 0.43, which was calculated for this stock, is close to that found for another population of pilchards in Australia (0.47; Joseph 1981) and also to that assumed for a spatial model for this fishery (0.45; Fletcher 1992). These catch curves have also shown that there is variation in the strength of recruitment among years with the relative number of recruits (2- and 3-year-old fish) present in the catch in any one year providing some warning concerning the future catch rate of the fishery up to 2 years in advance. This is proving to be a valuable tool for the provision of information to both the fishermen and fisheries' managers.

Notwithstanding some imperfections, the application of this otolith weight technique to provide estimates of age of pilchards for the Albany fishery has been most useful. More traditional methods of counting the annuli on otoliths of this species have proved difficult to implement owing to the presence of a number of ancillary rings on many otoliths that has substantially complicated the process of reading and validation (W.J. Fletcher and S.J. Blight, unpublished). As a consequence, the precision of the readings and in particular, the long-term reproducibility among readers, and readings by the same person, is only in the vicinity of 40–50% (W.J. Fletcher and S.J. Blight, unpublished data) which is a common problem in the ageing of *S. sagax* in South Africa (R. Crawford, personal communication) and California (P. Wolf, personal communication). Thus, despite the lack of precision using the otolith weight technique, its accuracy at the moment is at least as good as (e.g., similar growth curves have been generated by the two methods), and possibly superior to, the more time consuming and subjective method of counting rings.

It appears that the otolith weight technique can provide reasonable data on changes in the age composition of the catch of relatively short-lived species such as pilchards. The precision of the technique may improve when applied to species that have a single short spawning cycle per year because this should provide the clearest definition between age classes. Not all investigations have been able to show clear separation among ages using otolith weight (e.g., Beckman et al. 1991) and for longer lived species (>10 years), it is unlikely that age classes of just 1 year could be discerned and less precise groupings would have to be used. This would obviously limit the effectiveness of the otolith weight technique in discriminating yearly variations in recruitment (Bradford 1991). However, even ages provided by reading the annuli may be imprecise for such species, and a cost–benefit analysis could be done to determine which technique would be more reliable.

The ability to use otolith weight as a reliable method of ageing the catch of this species has enabled the first economical calculations of growth and mortality rates for this population which will greatly assist the management of the pilchard fishery in the Albany region of Western Australia. Similar studies on other stocks of pilchards in Western Australia and other local species are progressing and look promising but the extent to which otolith weight can be used as an estimate of age of an individual is likely to vary among species. Nevertheless, given the ease of measurement and the objective and consistent nature of each reading, the relationship between otolith weight and age should be explored as part of all studies on ageing.

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Sampling of the fish and otoliths was completed with the help of S. Blight, M. Rossbach and R. Tregonning. Thanks must also be given to the pilchard fishermen and factories of Albany and Fremantle for providing samples. Finally this manuscript benefited from comments by many of the staff at the Western Australia Marine Research Labs especially Dr. J. Penn and N. Hall along with comments from referees and Dr. J.D. Neilson.

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Distinguishing between resident and migrating Atlantic salmon (*Salmo salar*) stocks by genetic stock composition analysis

Marja-Liisa Koljonen

Abstract: The possibility of using the genetic stock identification (GSI) method to distinguish between individual Atlantic salmon (*Salmo salar*) stocks and stock groups in Finnish catches was studied. In the Baltic Sea, the Atlantic salmon is a target of a mixed-stock fishery, and information about stock composition would be valuable for the management of the species. The salmon catches on the Finnish west coast consist of two seasonally variable components: a group of northern stocks migrating through the area to the Baltic main basin and the resident Neva salmon. The migratory component includes two endangered wild stocks (Tornionjoki and Simojoki). The allele frequency differences at four polymorphic loci among the stocks allowed reliable catch composition estimates to be made of the migratory and resident components; one stock (Oulujoki) from the northern group could also be identified with reasonable accuracy. Northern migrating stocks accounted for over half the catches at the time of this study. The estimate of natural (nonhatchery) stocks was very low (3% in total).

Résumé : Nous avons étudié la possibilité de recourir à l'identification génétique des stocks (GSI) pour différencier les stocks et les groupes de stocks de saumon atlantique (*Salmo salar*) dans les prises débarquées en Finlande. Dans la Baltique, le saumon atlantique fait l'objet d'une pêche mixte et il serait utile d'en savoir davantage sur la composition des stocks pour assurer une bonne gestion de la ressource. Les prises de saumons sur la côte ouest de la Finlande sont constituées de deux groupes qui varient selon la saison : un groupe de stocks du nord, qui traverse la région dans sa migration vers le bassin principal de la Baltique, et le saumon local de la Neva. La composante migratrice comporte deux stocks sauvages menacés (Tornionjoki et Simojoki). Les différences observées entre les divers stocks dans la fréquence des allèles sur quatre loci polymorphes permettent d'obtenir des estimations fiables de la proportion des composantes migratrices et locales dans les prises. Un des stocks (Oulujoki) du groupe du nord pourrait également être identifié avec une exactitude raisonnable à l'aide de cette méthode. Les stocks migrateurs du nord représentaient plus de la moitié des prises à l'époque de la réalisation de cette étude. La proportion estimative des stocks naturels (non issus des écloseries) était très faible (3% au total).

[Traduit par la Rédaction]

Introduction

The Atlantic salmon (*Salmo salar*) is a target of a mixed-stock fishery in the Baltic Sea. The Finnish salmon catch offshore in the Bothnian Sea area has two main components: a relatively resident and temporally stable salmon stock and a group of northern migrating stocks. The resident salmon stock consists exclusively of fish from the Neva stock that are released into the Bothnian Sea and remain there throughout their lives (Kallio-Nyberg and Ikonen 1992). This stock was imported from the Neva, a Russian river draining into the Gulf of Finland (Fig. 1). There are no conservation needs to restrict fishing of the Neva stock in the Bothnian Sea.

The northern migratory component consists of salmon stocks from five Finnish rivers: Tornionjoki, Kemijoki, Simojoki, Iijoki, and Oulujoki. These stocks migrate from the Bothnian Bay through the Bothnian Sea to the Baltic main basin for feeding, and back the same way for spawning. Some salmon from these stocks remain in the Bothnian Sea instead of proceeding to the Baltic main basin. Thus, the northern component can be further subdivided into seasonally occurring distant migrants and more permanent local migrants within the Gulf of Bothnia. In addition to fish from the Neva stock, all northern stocks can potentially contribute to catches in the Bothnian Sea.

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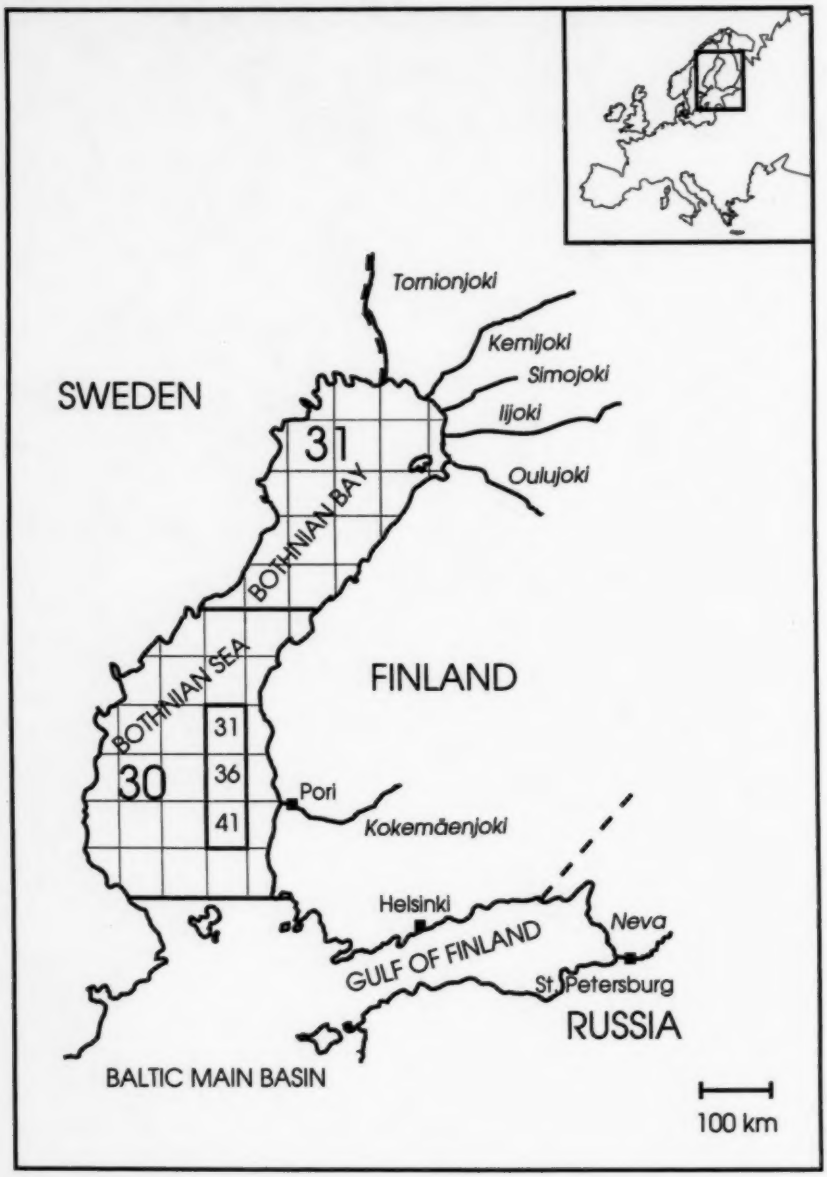
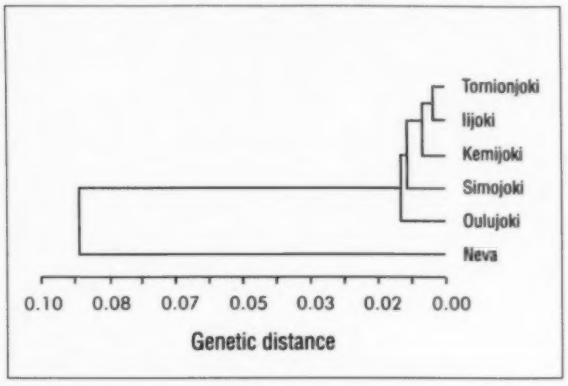
Fig. 1. Map of the study area, showing statistical rectangles sampled and the location of home rivers of the salmon stocks under study.



Only two of the five northern Finnish stocks (Tornionjoki and Simojoki) still reproduce naturally; they are also supported by stocking. These stocks are among the last wild Atlantic salmon stocks in the Baltic and are considered seriously endangered (Rassi and Väisänen 1987). Protection of these stocks is a major goal of salmon fishery management in the Baltic Sea. Fish from the wild stocks are expected to occur mainly in the far-migrating component. The stocks from the Kemijoki, Iijoki, and Oulujoki rivers no longer reproduce naturally owing to the construction of power plants, but they are cultured in hatcheries. The Iijoki and Oulujoki stocks are based mainly on the original native stocks, but no descendants from the original Kemijoki stock remain, and recent hatchery production is based on mixed brood of the Iijoki and Tornionjoki salmon. Swedish salmon stocks belonging to the migratory component may also contribute to Finnish catches.

Information on seasonal changes in the stock composition of salmon catches would be valuable for the management and conservation of the stocks, especially for drawing up

Fig. 2. Standard genetic distances (Nei 1978) between baseline stocks based on four polymorphic loci.



regulations for coastal fishing. In the present work, I studied the feasibility of using genetic stock identification (GSI) for estimating the proportions of different Atlantic salmon stocks in catches in the Bothnian Sea.

GSI has proved to be a useful method for estimating the proportions of different stocks in many mixed-stock fisheries for Pacific salmon (e.g., Milner et al. 1983; Gall et al. 1989; Shaklee et al. 1990*a*; Marshall et al. 1991). However, its usefulness is highly dependent on the amount of divergence among the stocks concerned. Statistically significant allele frequency differences can be observed among Finnish stocks of Atlantic salmon, but quantitatively the differences are small; only the Neva stock of Russian origin differs markedly (Koljonen 1989; Fig. 2).

If the differences among the stocks allowed sufficiently reliable estimates of the proportions of catch components to be made, considerably more information would be available than that produced by the more traditional Carlin tagging and scale analysis. Genetic stock composition analysis also has several practical advantages over Carlin tagging: no laborious marking is needed; the fish bear the genetic marks throughout their lifetime; fish that cannot be marked with Carlin tags or even with coded wire tags, e.g., wild fish and fish released as fry, can be examined (Murphy et al. 1983; Mathias et al. 1992); and the allelic differences have presumably no effect on the viability and catchability of the fish. In genetic stock identification, the time and place of sampling can also be chosen much more freely and precisely than with Carlin tagging. Moreover, genetic identification is cost effective and does not depend on whether or not fishermen return tags. The limitations of GSI are that there must be sufficient genetic differentiation among the contributing stocks and that it is generally not possible to obtain data on the origin of individual fish.

Scale analysis is another method used in estimating the stock composition of Atlantic salmon catches in the Baltic Sea. However, it is only useful for differentiating between wild and hatchery-produced fish released as smolts (Antere and Ikonen 1983; Hiilivirta et al. 1991). No distinction can be made among different hatchery stocks, and the usefulness of this method is limited by the fact that the number of wild Atlantic salmon in the Baltic is currently very small.

Table 1. Composition of baseline stock samples from the various rivers in the study area.

Stock	Sample	N
Tornionjoki: natural and hatchery production	Juveniles 1981	40
	Spawners 1981	20
	Juveniles 1992	260
	Total	320
Kemijoki: hatchery production	Spawners 1988	97
	Juveniles 1992	222
	Total	319
Simojoki: natural and hatchery production	Juveniles 1992	458
Iijoki: hatchery production	Spawners 1986	77
	Spawners 1987	54
	Juveniles 1990	180
	Juveniles 1993	153
	Total	464
Oulujoki: hatchery production	Juveniles 1981	48
	Juveniles 1992	180
	Total	228
Neva: hatchery production	Juveniles 1990	149
	Juveniles 1993	171
	Total	390
Total		2179

Materials and methods

Sampling of fish

For GSI, allele frequency estimates are needed of the catch mixture and also of the baseline stocks that potentially contribute to the mixture. Baseline samples were compiled over several years from salmon samples from the Tornionjoki, Kemijoki, Simojoki, Iijoki, Oulujoki, and Neva stocks (Table 1; partly published in Koljonen 1989). The 2179 fish in these samples comprised both spawners and juveniles, i.e., fish 1–3 years of age.

In general, the allele frequencies of different years were very similar, but significant temporal differences were observed in two cases: the Tornionjoki 1981 and 1992 samples differed in their *sAAT-4** frequencies ($\chi^2 = 28.10$, df = 2, $p < 0.001$) and the Neva 1990 and 1993 samples, in their *IDDH-2** ($\chi^2 = 38.60$, df = 2, $p < 0.001$) frequencies. In both, the more recent sample was a hatchery sample and had less variation than the earlier one.

The fit to Hardy–Weinberg expectations was tested for baseline stocks. Two of 18 tests showed statistically significant deviations from the expected values. One was in the Oulujoki stock at locus *IDDH-2** ($\chi^2 = 8.80$, df = 1, $p < 0.01$) and the other was in the Neva stock at locus *IDDH-1** ($\chi^2 = 44.76$, df = 1, $p < 0.001$). In both cases the deviation was a deficiency of heterozygotes.

Samples of the mixed-stock fishery were collected from a commercial drift-net fishery in the Bothnian Sea during a 17-day period from April 23 to May 9 in 1991. The samples

Table 2. Allele frequencies at studied loci in baseline stocks and in catch samples from different rectangles.

Locus and allele	Stock						Statistical rectangle			
	Tornionjoki	Kemijoki	Simojoki	Iijoki	Oulujoki	Neva	31	36	41	Total
*sAAT-4**										
N	288	316	392	345	171	170	267	342	388	997
100	0.889	0.783	0.671	0.803	0.755	0.692	0.751	0.734	0.731	0.737
80	0.000	0.002	0.001	0.000	0.020	0.079	0.049	0.060	0.050	0.053
50	0.111	0.215	0.328	0.197	0.205	0.229	0.200	0.206	0.219	0.210
*IDDH-1**										
N	260	317	458	446	227	320	267	341	388	996
100	1.000	1.000	1.000	0.994	0.978	0.925	0.964	0.962	0.968	0.965
10	0.000	0.000	0.000	0.006	0.022	0.075	0.036	0.038	0.032	0.035
*IDDH-2**										
N	217	317	426	411	214	320	267	342	388	997
100	0.698	0.751	0.635	0.618	0.586	0.166	0.528	0.434	0.514	0.490
72	0.302	0.249	0.365	0.382	0.414	0.834	0.472	0.566	0.486	0.510
*IDHP-3**										
N	222	317	434	435	199	316	267	342	388	997
100	0.991	1.000	0.999	1.000	0.857	0.979	0.966	0.965	0.974	0.969
116	0.009	0.000	0.001	0.000	0.143	0.021	0.034	0.035	0.026	0.031

Note: For each locus, sample size (*N*) differences from Table 1 are due to unsuccessful enzyme analysis of some individuals.

were from three statistical rectangles (31, 36, and 41) in ICES (International Council for the Exploration of the Sea) subdivision 30 (Fig. 1). The Neva salmon stocking occurs off Pori, near the mouth of the River Kokemäenjoki, adjacent to rectangle 36. According to data from earlier years, distant migrants arrived in the Bothnian Sea in June at the earliest (Anonymous 1992; Ikonen and Kallio-Nyberg 1993). Thus, the catches were assumed to represent a mixture of Neva salmon and short-distance migrants of the northern stocks only, which had not gone to the Baltic main basin at all.

Livers were taken by fishermen from 997 salmon while the catch was being processed. No muscle samples were available from these normally marketed fish. However, the most variable loci are known to be those active in the liver (Koljonen 1989).

Electrophoresis

Liver extracts were subjected to horizontal starch-gel electrophoresis. The nomenclature of the enzymes and loci conforms with the standardized system of the American Fisheries Society (Shaklee et al. 1990*b*) and, therefore, differs to some extent from that used in Koljonen (1989). Four variable loci were analysed: *sAAT-4** (AAT, aspartate aminotransferase, E.C. 2.6.1.1, formerly *AAT-4*); *IDDH-2** (IDDH, L-iditol dehydrogenase, E.C. 1.1.1.14, formerly *SDH-1*, sorbitol dehydrogenase); *IDHP-3** (IDHP, isocitrate dehydrogenase, E.C. 1.1.1.42, formerly *IDH-3*) and *IDDH-1**.

The buffer systems used were those of Cross and Payne (1977): 5 h, 10 V/cm for IDHP; and of Varvio-Aho and Pamilo (1980): 5 h, 10 V/cm for AAT and IDDH. The staining procedures were as reported by Allendorf et al. (1977) and Shaw and Prasad (1970). The designations for the variant alleles at *IDDH-2** and *IDHP-3** were changed to make them consistent with those in more general use (Verspoor et al. 1991).

Estimation

The estimates for stock composition were obtained by the conditional maximum likelihood method (Fournier et al. 1984; Pella and Milner 1987) using the GIRLSEM program (Masuda et al. 1991). Given the multilocus genotypic distributions in the c baseline stocks and in the observed catch sample, the method uses an iterative search algorithm to find the proportions of baseline stocks (p_i, $i = 1, 2, ..., c$, $\Sigma_{i=1}^{c} p_i = 1$) for such a stock mixture, from which the probability of obtaining the observed distribution of N genotypes in the catch would be the greatest.

The probability of sampling a particular multilocus genotype h from any mixture is $\lambda_h = \Sigma_{i=1}^{c} p_i g_{hi}$, when g_{hi} is the frequency of that genotype in the baseline stock i. The probability of the sample or the likelihood function being maximized with respect to the p_is is obtained as the product of the individual probabilities of the N observed genotypes:

$$L = \prod_h \lambda_h^{m_h}$$

where m_h is the observed number of fish of multilocus genotype h in the catch sample ($\Sigma m_h = N$). The baseline multilocus genotype frequencies used in the estimation process are calculated from the observed baseline allele frequencies assuming Hardy–Weinberg genotypic distribution and gametic phase equilibria among loci (Pella and Milner 1987). The search began at the point at which all the stocks contributed equally.

The stock composition estimates are subject to sampling errors in the genotype composition of both the baseline stocks and the catch samples. The standard errors due to both these samplings were estimated using the bootstrap

Table 3. Maximum likelihood estimates of proportions (%) of different stocks, bootstrapped estimates (average) and standard deviations resulting from resampling of mixture (SD_{mix}) and baseline (SD_{base}) data both separately and combined (SD_{tot}).

Rectangle	Dates	*N*	Stock	Sample Estimate (%)	Bootstrap			
					Average %	SD_{tot}	SD_{mix}	SD_{base}
31	Apr. 24–29	267	Tornionjoki	0.0	4.5	7.5	6.4	2.9
			Kemijoki	38.8	29.1	12.6	10.7	5.9
			Simojoki	0.0	2.5	5.3	4.1	1.6
			Iijoki	4.7	9.2	12.2	10.5	7.9
			Oulujoki	21.3	20.4	7.6	6.4	4.1
			Neva	35.2	34.3	5.0	4.7	2.3
36	Apr. 29 – May 9	342	Tornionjoki	0.0	1.8	4.2	3.7	1.5
			Kemijoki	0.8	4.7	7.0	6.5	4.2
			Simojoki	5.9	7.2	7.5	7.1	4.4
			Iijoki	27.5	19.6	12.9	11.2	8.1
			Oulujoki	24.1	21.5	6.8	5.5	3.8
			Neva	44.4	45.2	5.1	4.5	2.4
41	Apr. 23 – May 9	350	Tornionjoki	0.0	0.1	0.5	0.2	0.5
			Kemijoki	7.4	9.4	10.5	8.6	8.5
			Simojoki	7.5	8.9	9.2	8.0	6.4
			Iijoki	29.4	25.8	14.5	12.2	10.7
			Oulujoki	25.8	25.5	7.4	5.7	5.1
			Neva	29.9	30.3	4.9	4.0	2.7
Total	Apr. 23 – May 9	997	Tornionjoki	0.0	0.4	1.8	0.3	0.7
			Kemijoki	14.9	14.0	9.7	7.2	7.8
			Simojoki	3.2	5.2	5.6	4.4	4.4
			Iijoki	22.5	20.9	12.4	8.9	10.3
			Oulujoki	22.7	22.9	5.4	3.4	4.4
			Neva	36.7	36.6	3.6	2.7	2.5

resampling method (Efron 1982; Efron and Tibshirani 1986; Pella and Milner 1987). In this method the actual number of fish studied is resampled with replacement from the original sample, and the estimation process is repeated for each resampling. The errors resulting from the mixture and baseline sampling were evaluated both jointly and separately for both sources. The bootstrap estimate of bias is the difference between the sample estimate and the bootstrap average.

The proportion of a stock group can be estimated by summing the composition estimates for individual stocks within groupings based on stock similarity (the allocate and sum method; Wood et al. 1987). However, if the stocks to be combined are very similar, pooling of baseline data (the pool and allocate method) is recommended (Gall et al. 1989). In this study, the proportion estimated for the stock group of the four most similar stocks (Tornionjoki, Kemijoki, Simojoki, and Iijoki) was calculated both ways. The allele frequencies for the pooled baseline stock were calculated by weighting the allele frequencies of the different stocks with their smolt production proportions (see Discussion). Allele frequencies, genetic distances, and other basic genetic statistics were calculated using the BIOSYS-1 program (Swofford and Selander 1981).

Results

Differentiation among stocks and catch samples

The genetic divergence among the salmon stocks studied was generally low (Fig. 2). Neva salmon, which originate in the Gulf of Finland, clearly deviated from the others, all of which are from rivers discharging into the Bothnian Bay (Fig. 1). The largest differences were in the allele frequencies of the *IDDH-2** locus (Table 2). The discriminatory power of the individual loci can be measured using the variances of the allele frequencies (Gomulkiewicz et al. 1990), which were as follows: *sAAT-4**, 0.006; *IDDH-1**, 0.009; *IDDH-2**, 0.044; and *IDHP-3**, 0.003.

Identification of the Neva stock is based on allele frequency differences especially at *IDDH-2**, but also at *IDDH-1** and *IDHP-3**. The Oulujoki stock clearly deviates from the others in its *IDHP-3** frequency, where the variant allele **116* is more common than in the other stocks. In addition to these stocks, only the natural stocks of Tornionjoki and Simojoki showed variation at *IDHP-3**, and the Iijoki stock at *IDDH-1**.

The same alleles occurred in the catch samples as in the baseline data, and all the mixture genotypes could have arisen from the baseline stocks, i.e., no novel genotypes

Table 4. Estimated proportions (%) of two stocks (Neva and Oulujoki) and a stock group of the four most similar northern stocks in the three statistical rectangles.

Rectangle	*N*		Summed estimate (%)	Bootstrap Average (%)	Bootstrap SD	Sample estimate (%)	Bootstrap Average (%)	Bootstrap SD_{tot}
31	267	Stock group	43.5	45.4	6.6	45.9	45.4	5.9
		Oulujoki	21.3	20.4	7.6	19.6	20.2	7.4
		Neva	35.2	34.2	5.0	34.5	34.4	4.9
36	342	Stock group	34.2	33.2	6.6	29.0	28.6	5.8
		Oulujoki	21.4	21.5	6.8	22.6	23.1	7.1
		Neva	44.4	45.3	5.1	48.4	48.3	4.9
41	350	Stock group	44.3	44.1	6.9	39.4	39.5	6.5
		Oulujoki	25.8	25.6	7.4	27.0	27.1	8.4
		Neva	29.9	30.3	4.9	33.6	33.4	4.7
Total	997	Stock group	40.6	40.5	5.0	37.9	37.6	4.1
		Oulujoki	22.7	22.9	5.4	23.2	23.3	5.6
		Neva	36.7	36.6	3.6	38.9	39.1	3.4

Note: The summed estimate is the allocate and sum estimate, and the sample estimate is the pool and allocate estimate. Bootstrapped averages with their standard deviations are given for both estimates. Both mixture and baseline data were resampled for SD_{tot}.

occurred in the catches (Table 2). The allele frequencies of the three catch samples were similar. No statistically significant difference was observed between the samples from rectangles 31 and 41, but the sample from rectangle 36 differed slightly from the others ($\chi^2 = 11.53$, df = 5, $p < 0.05$ and $\chi^2 = 11.69$, df = 5, $p < 0.05$, respectively).

Stock composition estimates

Stock composition was estimated for each statistical rectangle separately, and for the combined data (Table 3). The resident catch component, the Neva salmon, was the most abundant individual stock in the total catch in this study. The Neva stock accounts for at least one third of the catch in all three rectangles, and in rectangle 36, adjacent to the release site, for almost half (44%, Table 3 and 48%, Table 4).

Within the northern component, the Oulujoki stock regularly constituted about 20% of the catch at the time of this study. Of the other four stocks, the hatchery stocks from the Kemijoki and Iijoki rivers were quite abundant and together accounted for a substantial proportion of the total catch. These stocks were the only ones that showed some differences among the rectangles (Table 3). The proportion of natural stocks (Tornionjoki and Simojoki) was very small, and their joint estimate was only 3% of the total catch.

The reliability of the estimates evaluated with bootstrapping depended on the stock concerned. Estimates for the Neva and Oulujoki stocks were the most reliable. The estimated bias (differences between sample estimate and bootstrap average) were insignificant (Table 3) and the relative errors of the estimates were at a reasonable level. The results for these stocks were already quite reliable with a sample size of 300 from individual rectangles. The errors in the estimates of the two natural stocks were marked, but because of their small contribution to the catch, the total error resulting from these estimates was small.

The absolute errors were greatest in the proportions of the Kemijoki and Iijoki stocks, which reflects the difficulty in distinguishing these stocks from each other, and also their abundance in the catch. A negative correlation in their estimates was evident.

The four northern stocks (Tornionjoki, Kemijoki, Simojoki, and Iijoki) are genetically so similar that it was impossible to identify their individual proportions reliably; therefore, the estimates are only indicative. When these four stocks were pooled, the error for the stock group was smaller than that for individual stocks on average (Table 4). The proportions estimated with the pool and allocate method and the allocate and sum method were very similar (Table 4), but the mean errors were slightly smaller for the pool and allocate estimate than for the allocate and sum estimate for the four-stock group.

The source of the variation was analysed by bootstrapping the mixture samples and baseline samples separately. In the separate rectangle samples (300 fish), the error resulting from sampling of the mixture (SD_{mix}) was larger than that from baseline data (SD_{base}). In the combined sample (1000 fish), the error was lowered to the same level as in the baseline sampling.

The stock proportion estimates were evaluated by comparing the actual mixture sample allele frequencies (counts) with an expectation derived by mixing the baseline frequencies in the estimated stock proportions using a χ^2 goodness-of-fit test. The observed and expected frequencies differed ($p < 0.05$) from each other at the *sAAT-4** locus

Table 5. Absolute and relative smolt production in baseline stocks in 1989 and estimated stock proportions in the catch from the Bothnian Sea in late April and early May 1991.

Stock	Production				Estimated GSI (%)
	Natural	Hatchery	Total	%	
Tornionjoki	75 000	15 000	90 000	5.2	0.0
Kemijoki	—	759 000	759 000	44.1	15.0
Simojoki	10 000	82 000	92 000	5.3	3.2
Iijoki		293 000	293 000	17.0	22.5
Oulujoki	—	241 000	241 000	14.0	22.7
Neva	—	247 000	247 000	14.4	36.6
Total	85 000	1 637 000	1 722 000	100	100.0

with an χ^2 value of 9.03 (df = 2) and at all four loci with a value of 13.94 (df = 5). The clearest difference was in the frequency of allele **80* of the *sAAT-4** locus, which was more common in the actual mixture sample than in the estimated mixture. The frequency of this allele is highest in the Neva salmon (Table 2).

Discussion

Catch proportions

On the basis of the genotypic data from four polymorphic loci, it was possible to identify the components of resident and migrating salmon in Finnish Atlantic salmon catches. The resident component consisted exclusively of the Neva stock, whereas the migrating component comprised five different migrating stocks. The estimates of these two major groups were reliable enough for practical purposes. In addition, the proportion of the Oulujoki stock within the northern component could be estimated relatively reliably. Estimates of the relative proportions of the remaining four stocks should be regarded as only approximate.

The northern stocks constituted over half the catches (55–70%) in the Bothnian Sea, even before the main spawning migration season in early spring. This suggests that a marked proportion of northern fish remained in the Bothnian Sea. The extent to which different stocks tend to remain in the Bothnian Sea is unknown, and depends on both the size of the released smolts and the food available to them each year. Because of an earlier shift to fish as food, larger smolts are more likely to end their migration in the Bothnian Sea (Salminen et al. 1994). The released smolts tend to be larger than wild smolts on average, and thus, a greater proportion probably remain in the Bothnian Sea.

Estimates of individual stocks in the Bothnian Sea can be compared with their relative smolt production, which is the average proportion of each stock in the sea. The majority (68%, March 1 – May 9 in the study area, on the basis of Carlin tag data) of the catches in 1991 were from the 1989 smolt year-class, which has thus been used as a reference year (Table 5). The contribution of the Neva stock is clearly larger than that expected from the number of stocked smolts; moreover, the proportion was about 10% higher in the rectangle adjacent to the release site. The sampling area, the Bothnian Sea, is the basic feeding and stocking area for this stock, and thus, local overrepresentation is expected.

Among the northern stocks, some overrepresentation was observed in the estimates for the Oulujoki stock, in relation to smolt production. This may be due to the different tendencies of stocks to remain in the Bothnian Sea or to movements of salmon schools in the area. The Iijoki and Kemijoki stocks resembled each other so closely that it was impossible to evaluate their individual contributions very precisely, but together they made up a large proportion of the total catch. In combination, these stocks were represented by a large proportion of the smolt production as well.

The low estimated proportions of catches of both the Tornionjoki and Simojoki natural stocks were consistent with their small smolt production proportions. It is unlikely that the proportion of wild stocks is large at any time during the fishing season, although it is probably slightly larger when the far-migrating component reaches the coast. The greatest changes are both possible and expected in the proportions of the largest stocks, as changes in small proportions are difficult to observe. The great difficulty in conserving and managing Atlantic salmon in the Baltic is due to the very small proportion of wild fish in the total population. It is worth determining whether GSI might be a suitable tool for obtaining more detailed information on seasonal changes in the proportions of wild stocks.

Reliability of the estimates

Errors in the composition estimates may derive from the mixture sample, the baseline sample, or both. When the sample sizes are large enough, these two errors are additive in theory (Pella and Robertson 1979; Millar 1987). The bias, however, is mainly derived from the baseline data (Pella and Robertson 1979) and is greatest when genetically similar stocks differ greatly in abundance (Millar 1987).

The variation resulting from mixture sampling depends on the sample size and stock composition of the sample. Wood et al. (1987) observed that the number of stocks contributing to the mixture has little or no effect on the model performance, although the number of fish per stock sample is important. If, however, the number of contributing stocks is large, larger mixture samples are needed for reliable estimates. According to Wood et al. (1987), the critical sample size for several levels of separation is about 40 fish

per stock; below that the reliability of the estimates is greatly reduced. In general, it was clear that with finite mixture samples, the model underestimated contributions by dominant stocks and overestimated contributions by minor stocks. The sample size of 300 used in this study was sufficient for distinguishing among the three major components.

Baseline sampling is in general more problematic than mixture sampling. Several factors may affect the amount of variation and bias in the estimates: the completeness of the baseline data, conformity to Hardy–Weinberg expectations, temporal variation in allele frequencies, sample sizes, number of loci, genetic differentiation among stocks, and pooling strategies.

An important question is whether all the contributing stocks were represented in the baseline data (Smouse et al. 1990). All Finnish stocks were included in the baseline data, but the occurrence of Swedish salmon in the Finnish catches was also possible. Owing to this possibility, an additional test was conducted to check for the presence of Swedish salmon. Data for three loci, *sAAT-4**, *IDDH-2** and *IDHP-3** on salmon from eight Swedish rivers were available for this purpose (Ståhl 1981, 1983, 1987; M.-L. Koljonen unpublished). However, no contribution of Swedish stocks was detected (1% of one stock). However, the result for Finnish stocks alone, based only on data for these same three loci, differed slightly from the results based on the four Finnish loci: the proportion of the Neva stock increased from 37% to 41%, that of the Oulujoki decreased from 23% to 18%, and the contribution of the Iijoki stock was transferred to that of the Kemijoki stock, which made it increase to 40%. The contribution of the three main components (Neva, Oulujoki, and the rest of the stocks) nevertheless remained quite similar.

Although no contribution of Swedish stocks was observed, their allele frequencies (Ståhl 1981, 1983, 1987) are so similar to those of the northern Finnish stocks (other than the Neva and Oulujoki) that they are most likely included in the proportion estimate of the stock group. Moreover, on the basis of Swedish Carlin tags returned to Finland by Finnish fishermen, the proportion of Swedish fish in the catches was estimated to be 9% at the time and in the area of this study.

Errors in baseline sampling can also arise if individual stocks are composed of several breeding units, either natural or hatchery units, or if variation in the allele frequencies of different year-classes is marked. This may cause deviations from the Hardy–Weinberg distributions in the stocks and may also mean that the allele frequencies observed are not representative of the stock as a whole. Such problems are most likely to arise in small natural stocks or in hatchery stocks with changing brood fish and breeding ranges.

In the present baseline data, the genetically most stable stocks are probably those of the Iijoki, Oulujoki, and Neva, whose production is consistently based on the same broodstock lines. The Tornionjoki and Simojoki stocks are produced both naturally and in hatcheries, and analysing these components separately or weighting their allele frequencies with their production relations might have some effect on the results.

The estimated bias was greatest in the proportion of the Kemijoki stock, and the negative correlation between the estimates for the Kemijoki and Iijoki stocks indicates that it might be sensible to pool these stocks (Millar 1987). The similarity of these stocks is understandable, in view of their partly common origin.

Significant deviations from Hardy–Weinberg equilibrium were observed in the Oulujoki and Neva stock baselines, so estimation was also carried out without assuming Hardy–Weinberg equilibrium. In this less precise but more robust method, the baseline data are handled as simple observed frequency data. However, the stock apportionment was exactly the same, so Hardy–Weinberg deviations evidently did not have any effect on the result in this case. In the case of the Oulujoki stock, the deviation was probably a result of the pooling of different year-classes (1981 and 1992), although a slight deficiency of heterozygotes could be observed in the 1992 sample as well, at the *IDDH-2** locus ($\chi^2 = 5.55$, df = 1, $p < 0.05$). In the case of the Neva stock, the deviation could be observed only in the sample from 1990 (not in that of 1993). However, one possible explanation for the deviation in the 1990 sample might be the especially difficult interpretation of the *IDDH-1** locus.

Waples (1990) analysed the implications of temporal allele frequency changes for mixed-stock analyses and observed that, in general, temporal changes should be taken into account as potential sources of error. Any effects of changes caused by genetic drift can be compensated for to a considerable extent by collecting baseline data over several years. The importance of repeated sampling depends on the life history of the species concerned and on the degree of overlap in the year-classes. The Atlantic salmon has overlapping generations, and partly because of that, temporal variation in allele frequencies in large natural stocks is probably insignificant; although in small natural stocks or in hatchery stocks, genetic drift can cause marked changes.

Here, the baseline data were based on samples over several years in all but the Simojoki stock. In general, the differences between year-classes were insignificant, implying that temporal changes in allele frequencies are not large. There were, however, two exceptions: the Tornionjoki stock and the Neva stock. The case of the Tornionjoki stock is understandable, as changes in allele frequencies are very likely in a 10-year period in a relatively small stock with several different reproductive units. The significant difference in frequency between the 1990 and 1993 Neva stock year-classes is more unexpected and indicates that temporal changes can occur even when production is relatively stable. The difference may be the result of partial reproductive isolation of different year-classes (Waples 1989), although it may also be attributable to the difficulty in obtaining representative samples of juveniles. The stock composition analysis was also carried out using only the more recent sample of the Tornionjoki, Neva, and Oulujoki stocks, but the stock apportionment remained the same after these changes.

According to Wood et al. (1987), a critical value for the baseline sample size is about 50 fish per stock, a level that was clearly attained here. The number of fish needed per stock, however, also depends on the level of genetic differentiation among the stocks. When evaluated in the

light of the bootstrap results, the number of fish sampled in this study was sufficient both in the baseline data and in the mixture sample.

Both the number of loci and the level of differentiation between the stocks affect the discriminatory power of the baseline data. Seven polymorphic loci are known for Atlantic salmon in the Baltic Sea and including them all in the analysis should improve the accuracy (the three variable muscle loci are *sMDH-3,4**, *mMEP-2** and *PGM-1**). The number of loci that could be analysed in this study was not large, when compared with those analysed in studies of Pacific salmonids (e.g., Shaklee et al. 1990*a*, 14–22 loci; Brodziak et al. 1992, 15 loci). More than 10 loci is probably impossible to attain with Atlantic salmon, but even with fewer loci, useful results can be obtained.

Separation among the genetically most similar stocks was relatively limited. In the event of small separation, performance of the analysis can be improved by increasing the number of loci or the mixture sample size; however in some cases, the pooled estimate is the only one that can be obtained reliably.

Both pooling methods (pool and allocate and allocate and sum) have their advantages. Wood et al. (1987) recommended that the allocate and sum method be used when the stocks to be pooled are not very similar. Gall et al. (1989) recommend the pool and allocate method if the members of a stock group have relatively homogeneous genetic profiles or if the mixture samples are small. Brodziak et al. (1992) compared the performance of these two methods and obtained very similar results with both. In the present work as well, the estimates were calculated both ways without revealing any significant differences.

GSI proved to be a sufficiently reliable method for distinguishing between resident and migrating components of Atlantic salmon in the Baltic Sea. It also seems to be a feasible method for obtaining in-season estimates of these components.

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Influence of prehatch temperature on the development of muscle cellularity in posthatch Atlantic salmon (*Salmo salar*)

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Abstract: Previous work has shown that higher incubation temperatures produce newly hatched salmon (*Salmo salar*) with fewer but larger muscle fibres than salmon incubated at colder temperatures. Our purpose was to study the effect of differing incubation temperatures on the development of muscle cellularity in posthatch salmon. Eggs from a single pair of Atlantic salmon were incubated at either the stream ambient temperature (fluctuating around 5°C prehatch and gradually rising to around 10°C posthatch) or at 11°C. From each group, samples were taken at hatching, first feeding, and at 3 weeks after first feeding. During the period of exogenous feeding under study, the ambient group grew faster. The number of muscle fibres remained lower in the 11°C fish but there was more muscle fibre hypertrophy in the ambient group so that the difference in muscle fibre size seen at hatching was eliminated by 3 weeks after first feeding. It is suggested that reduced number of nuclei in the 11°C fish at hatching may contribute to the relatively reduced fibre hypertrophy in these fish. The results indicate that embryonic myogenesis can affect the posthatch growth of salmon at least up to 3 weeks after first feeding.

Résumé : Des travaux antérieurs ont démontré que les températures d'incubation plus élevées produisent des alevins de saumon atlantique (*Salmo salar*) pourvus de fibres musculaires moins nombreuses, mais plus grosses, comparativement aux saumons incubés à des températures plus basses. Nous avons examiné l'effet d'une variation des températures d'incubation sur le développement des cellules musculaires des saumons, après l'éclosion. Les oeufs d'une seule paire de saumons atlantiques ont été placés à incuber soit à la température ambiante du cours d'eau (oscillant aux alentours de 5°C avant l'éclosion et augmentant par la suite graduellement jusqu'à 10°C), soit à 11°C. Des échantillons ont été prélevés dans chaque groupe à l'éclosion, au début de l'alimentation, et 3 semaines plus tard. Pendant la période de nutrition exogène faisant l'objet de l'étude, le groupe placé à la température ambiante a montré une croissance plus rapide. Le nombre de fibres musculaires est demeuré inférieur chez les poissons élevés à 11°C, mais les fibres étaient plus grosses chez les sujets placés à la température ambiante, et la différence observée dans la taille des fibres musculaires au moment de l'éclosion avait disparu 3 semaines après le début de l'alimentation. Il est possible que le nombre réduit de noyaux, au moment de l'éclosion, chez les poissons placés à 11°C, ait contribué à la réduction relative de l'hypertrophie des fibres chez ces poissons. Ces résultats démontrent que la myogenèse peut influer sur la croissance des jeunes saumons au moins jusqu'à 3 semaines après le début de l'alimentation.

[Traduit par la Rédaction]

Introduction

Increasing temperature within the thermal range of a species can speed up embryonic development. The development of Atlantic salmon (*Salmo salar*) embryos at 10°C can be twice as fast as that of embryos incubated at 5°C (Usher et al. 1994). This fact can be used in salmon hatcheries to increase production and start the posthatch growing season earlier (Farmer et al. 1990). There is some evidence to suggest that an optimum temperature regime for the survival of fish embryos is one that is more closely related

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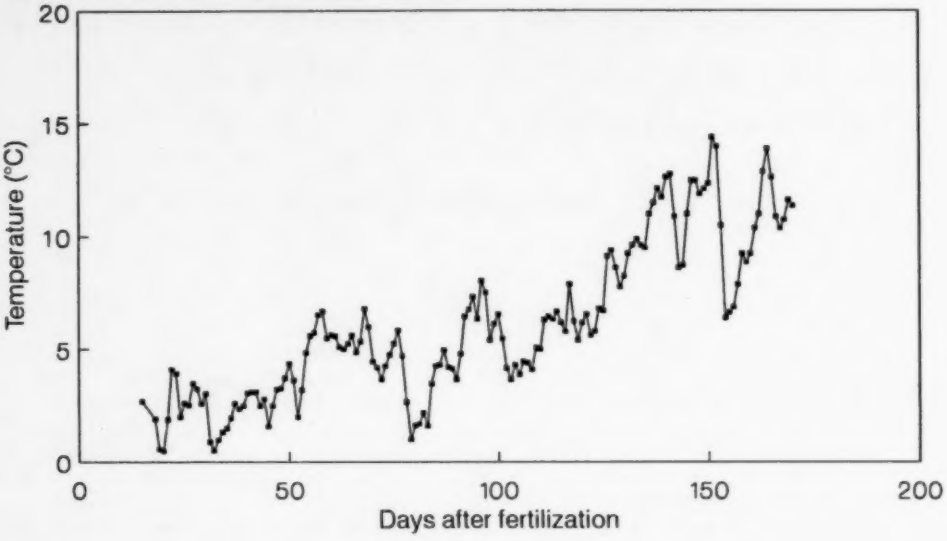
Fig. 1. The ambient temperature of Almondbank salmon hatchery from the day of fertilization up to 3 weeks after first feeding for the ambient group.



to the natural environmental conditions of embryogenesis for each species (Hokanson and Kleiner 1974; Luczynski 1985). During embryonic development, temperature can affect the organogenesis and meristic features. Although the rate of development and growth is increased, the number of vertebrae (Klinkhart et al. 1987) and the total number of muscle fibres (Stickland et al. 1988) on hatching are decreased at high incubating temperature. By contrast in older fish, faster growth is associated with increased muscle fibre hyperplasia (Weatherley and Gill 1987). Such correlation is less evident in very young posthatch stages of salmonoid species (Weatherley et al. 1980; Higgins and Thorpe 1990) and is probably due to less muscle fibre hyperplasia in young stages of some fish (Kiessling et al. 1991).

In fish, somatic growth is mainly a function of skeletal muscle growth. If early posthatch growth is mainly a result of muscle fibre hypertrophy, then larvae with larger and fewer muscles fibres may have less potential for hypertrophy compared with larvae with more smaller muscle fibres. Furthermore, any muscle fibre hyperplasia and hypertrophy that may occur in posthatch fish larvae could be influenced by the density of muscle nuclei. Salmon embryos incubated at higher temperatures produce hatched larvae with a lower density of nuclei in the muscle tissue (Usher et al. 1994). In view of this profound effect of temperature on myogenesis of embryonic salmon and the correlation between fish growth and muscle fibre growth dynamics, the aim of the present study was to investigate the effect of incubating temperature on the growth and muscle cellularity of posthatch stages of Atlantic salmon.

Materials and methods

Fish

All eggs were the progeny of a single pair of Atlantic salmon (one 2+ female and one 1+ male) that originated from the river Almond. The fertilized eggs, larvae, and fry were maintained at the Almondbank salmon hatchery station, DAFFS, Freshwater Fisheries Laboratory, Pitlochry, Scotland, U.K. After fertilization, the eggs were divided into two groups; each was placed on Perspex salmon egg incubating trays kept in plastic tanks. One group was kept at the ambient river temperature of the Almondbank hatchery (where the mature salmon of the local stock would spawn). The temperature of the other group was gradually raised to 11°C over 6 days and then maintained at the temperature throughout the experiment.

Ambient temperature was considerably lower than 11°C until the hatching of the ambient incubated fish embryos, at which time the temperature gradually increased such that at first feeding the river temperature fluctuated around 10–11°C (Fig. 1).

Measurements of fish size

From each group samples were taken at hatching (when 50% of eggs hatched), first feeding, and 3 weeks after first feeding. From each group five fish per stage were overanaesthetized in a solution of ethyl *m*-aminobenzoate methanesulfonate (MS-222). Length was measured to the nearest 1 mm. The total length was recorded on hatching, whereas for the other stages the fork length was recorded (Peterson et al. 1977). The fish were blotted dry with filter paper and the body weight, without the yolk for the hatched larvae, was recorded to the nearest 0.1 mg using a precision electronic balance (Sartorius 1712). During sampling it was observed that, at first feeding, the fish from the ambient group were similar in weight to the 11°C fish 3 weeks after first feeding. It was originally planned to take muscle samples only at hatching and at 3 weeks after first feeding for each group. However, to examine a possible correlation between fish body mass and muscle cellularity, muscle

Table 1. Wet weight (BW), length, and condition factor of developing salmon larvae.

Developmental stage	Temperature	Days from fertilization	Weight (BW) (mg) $n = 5$	Length (cm)	Condition factor (BW/L^3) ($g \cdot cm^{-1} \times 100$)	Specific daily growth rate (%)
Hatched	Ambient	117	39 (0.58)	2.0	0.48	
Hatched	11°C	40	29.14 (1.48)	1.9	0.42	
First feeding	Ambient	147	102.6 (0.24)	2.5	0.65	3.22
First feeding	11°C	80	76.6 (8.39)	2.3	0.62	3.23
3 weeks after first feeding	Ambient	168	204.2 (5.81)	2.9	0.83	2.40
3 weeks after first feeding	11°C	101	94.2 (1.13)	2.5	0.60	1.00

Note: Numbers in brackets indicate the standard error of the mean (SE). The SE of length is not given because it is less than 1 mm which was the precision of the measurements at each stage. Specific daily growth rate was estimated using the equation $(\ln(BW t_2) - \ln(BW t_1))/\text{days} \times 100$.

samples were also taken from the ambient group fish at first feeding.

Morphometry of myofibres

The whole body posterior to the vent was mixed and embedded according to Stickland et al. (1988). Transverse sections of 0.5-μm thickness were obtained using a Reichert ultramicrotome. The sections were treated with a saturated solution of ethanolic KOH for partial removal of resin and stained according to Ontell (1974). All measurements were made using a computerised image analysis system (Seescan Ltd., U.K.) linked to an Olympus (Olympus BH-2) light microscope. The total cross-sectional area of white muscle was traced on the screen for one side of the transverse section. Care was taken to exclude fin muscle fibres and the skin. The total number of white muscle fibres on this one side of the section was counted using high magnification. The cross-sectional area of individual white muscle fibres ($n > 200$) from the centre of the upper epaxial region of each side of the myotome as used by Stickland et al. (1988) was traced manually and recorded. The number of nuclei per cross-sectional area of white muscle was counted for each fish in five sample areas of the upper epaxial muscle, each of 6000 μm^2, using oil immersion light microscopy. The significance of differences in the measured parameters was determined by Student's *t*-test between the two groups.

Results

Embryos incubated at a constant temperature of 11°C hatched considerably earlier than those at ambient temperature (Table 1). The hatched larvae of the ambient group were significantly ($P < 0.002$) heavier than the 11°C group.

At 3 weeks after first feeding the ambient-reared alevin had more than twice the weight of the 11°C counterparts and their condition factor was higher (Table 1).

On hatching, the total white muscle cross-sectional area was not different between the two groups (Fig. 2*a*) while the myofibres of the ambient group were significantly ($P = 0.001$) smaller compared with the myofibres of the 11°C group (Fig. 2*b*). Consequently, the number of myofibres was significantly higher ($P = 0.007$) in the ambient group (Fig. 2*c*).

At 3 weeks after first feeding, the total muscle cross-sectional area was larger ($P = 0.014$) in the ambient group (Fig. 2*a*), but the mean size of white muscle fibres was not significantly different between fish of the two groups (Fig. 2*b*). Moreover, there is no evidence of different rates of muscle fibre hyperplasia, as seen by muscle fibre size–frequency distributions (Fig. 3). However, the total number of myofibres was significantly higher ($P = 0.021$) in the ambient group (Fig. 2*c*).

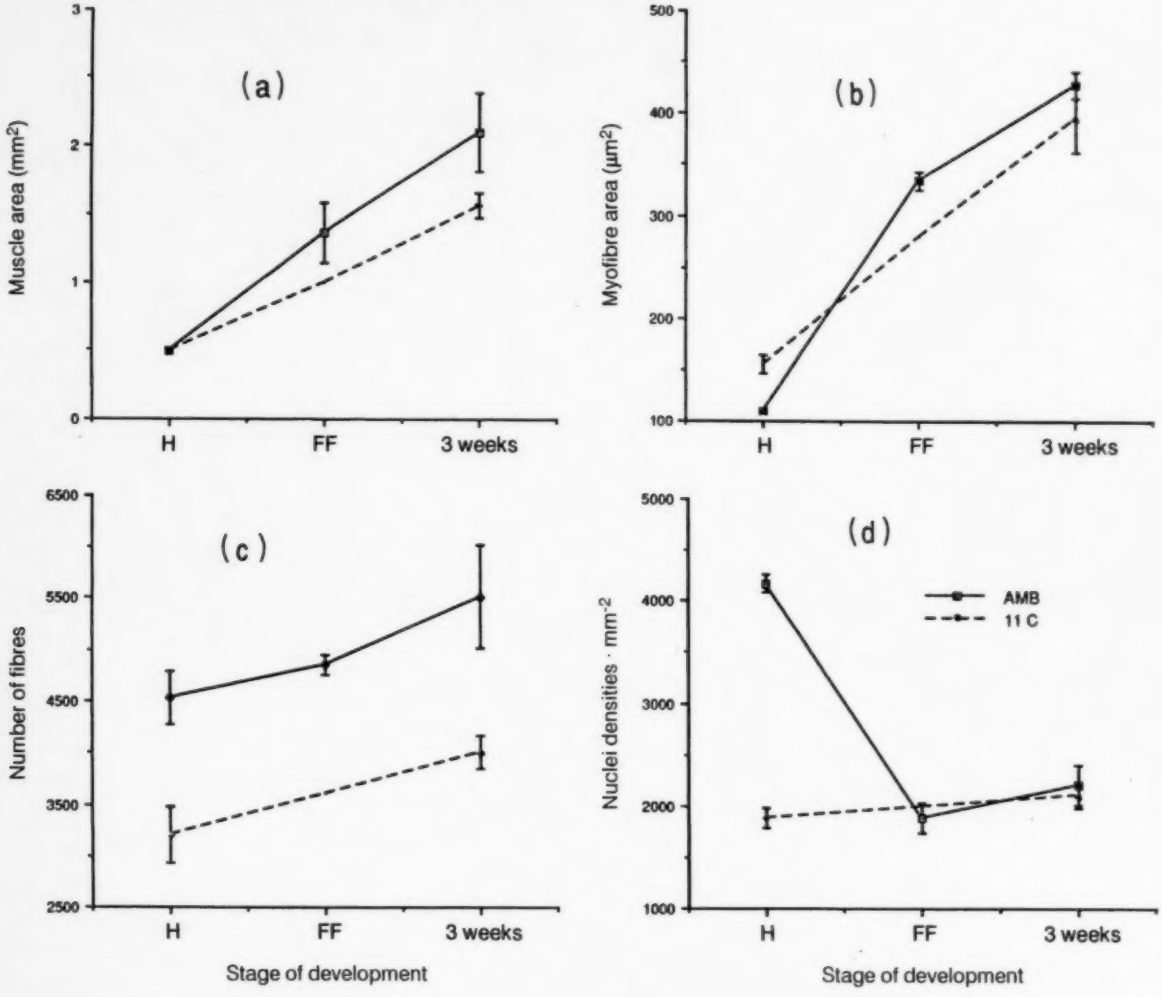
On hatching, the density of nuclei per cross-sectional area of white muscle was significantly higher ($P < 0.001$) in larvae of the ambient group, but subsequently the density of nuclei became similar in the two groups (Fig. 2*d*).

Discussion

Results indicate that incubating temperature can have a significant effect on posthatch larval growth at least up to 3 weeks after first feeding. Temperature had an accelerating effect on the growth rate of embryonic stages of development. Although the embryos incubated at a constant temperature of 11°C hatched considerably earlier than those at ambient temperature (Table 1), the hatched embryos that were incubated at the ambient temperature tended to be longer and heavier (Table 1). Peterson et al. (1977) also concluded that incubating salmon embryos at 6–7°C and then raising the temperature to 8–12°C after hatching, resulted in improved growth and larger size at first feeding than embryos and larvae reared constantly at 12°C throughout development. The relatively small difference in the size of hatched larvae became more obvious by first feeding and further increased after exogenous feeding (Table 1). This is in agreement with Beacham et al. (1985) who found, in other species of salmon, that small differences in size of embryos at hatching were enlarged by first feeding. Larval metamorphosis and transition from endogenous to exogenous feeding seems to dramatically change the body weight to length ratio in the ambient group (Table 1). The weight of the developing salmon tends to increase more rapidly than length after larval metamorphosis.

First feeding occurred at 32 days after hatching in the ambient group, whereas in the 11°C group, first feeding occurred at 40 days after hatching (Table 1). Because the

Fig. 2. Muscle growth and development of salmon reared at the ambient (solid lines) thermal regime and at 11°C (broken lines) on hatching (H), first feeding (FF), and 3 weeks after first feeding (3WKS). In each figure the bars indicate the standard error of the mean. (a) Total white muscle cross-sectional area (mm^2). (b) Mean myofibre cross-sectional area (μm^2) of white epaxial muscle. (c) Total number of white muscle fibres. (d) The density of nuclei per cross-sectional area of white muscle. Nuclei densities are adjusted for a cross-sectional area of 1 mm^2.

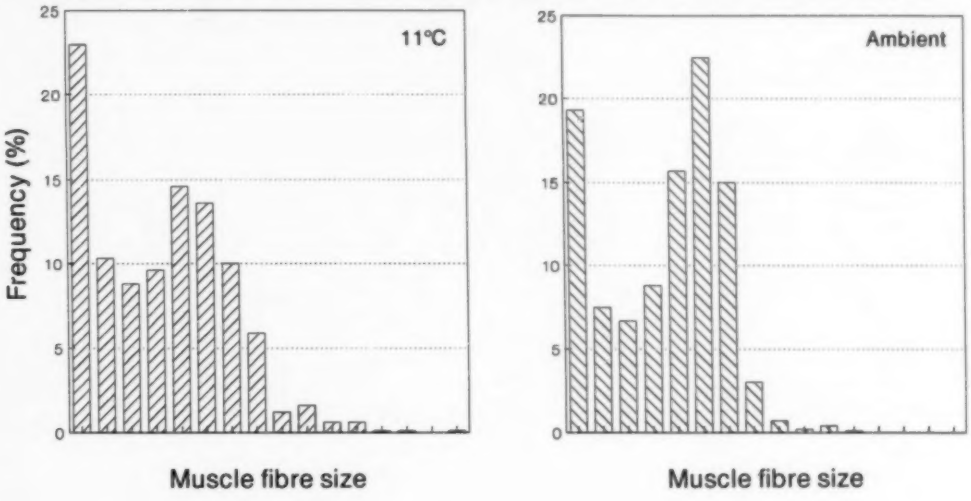


ambient temperature was gradually rising so that the mean temperature between hatching and first feeding was 9.7°C, and between first feeding and 3 weeks after first feeding it was 10.6°C, this difference in length of time between hatching and first feeding must have been a result of the incubation temperature and not of the posthatch temperature regimes. Furthermore, it should be stressed that the timing of first feeding does not coincide with the stage of 0% yolk. There is in fact a period of mixed endogenous and exogenous feeding (Kamler 1992). The faster growth and development of the ambient group probably occurred because this stock was adapted to the natural temperature regime of the river Almond. Such a growth difference indicates that the ambient thermal regime was the optimum for growth and development of the salmon used in this study.

The effect of incubation temperature on the development of posthatch salmon seen in the present study and also by Peterson et al. (1977) may be explained by the effect of temperature on muscle fibre hyperplasia and hypertrophy. On hatching there were more muscle fibres in the ambient group and the difference was similar to that found in hatched salmon incubated at ambient and 10°C (Stickland et al. 1988). At first feeding, the ambient group had more muscle fibres than the 11°C group had at 3 weeks after first feeding (Fig. 2*c*).

There is some evidence that muscle fibre hyperplasia is relatively low in young stages of development in rainbow

Fig. 3. The size–frequency distribution, expressed as percentages, of the white muscle fibres in salmon for the ambient and the 11°C group 3 weeks after first feeding. (n = 5 fish from each group).



trout (*Oncorhynchus mykiss*) (Weatherley et al. 1980; Kiessling et al. 1991) and Atlantic salmon (Higgins and Thorpe 1990). In this study also, muscle fibre hyperplasia was low in both groups (Fig. 2*c*). At hatching, larvae of the ambient group have smaller but more numerous muscle fibres and more nuclei (Figs. 2*b*–2*d*) compared with the larvae of the 11°C group. The smaller muscle fibres may exhibit a more optimum size for the diffusion of metabolites and greater potential for further growth. The relatively fewer nuclei in the 11°C larvae at hatching compared with ambient larvae may also be a contributory factor to the lower hypertrophy or hyperplasia, in that nuclei are required for both processes. As far as muscle fibre hyperplasia is concerned, more nuclei are required for the formation of new myofibres through fusion of precursor cells to form myofibres. Equally important is the number of nuclei for hypertrophic growth of muscle fibres because more nuclei are required to maintain an optimum ratio of nuclei to cytoplasm.

Salmon embryos are not active until towards the end of the endogenous feeding period and consequently muscle fibres are functionally more important after larval metamorphosis. In wild stocks, both larval growth and survival are critical for determining the recruitment in a stock, and directly affect the strength of the first year class (Gulland 1965). Larger size on first feeding can result in improved swimming performance, which will enhance active feeding (Blaxter 1969) and survival (Kazakov 1981; Wallace and Aastord 1984; Pepin and Myers 1991) of the exogenously feeding salmon larvae. This investigation has demonstrated that embryonic development of salmon at high incubation temperatures can have significant negative consequences on the muscle development, the larval size at first feeding, and consequently the viability of posthatch salmon. However, the results of this study were obtained with a Scottish Atlantic salmon stock and may not apply to other stocks of Atlantic salmon. Different stocks spawn at different seasons and environmental conditions and may, therefore, be adapted to their own particular environment. For example, there is some evidence that the effect of temperature on the rate of development of seasonally distinct spawning stocks of chum salmon (*Oncorhynchus keta*) may vary (Tallman 1986). Nevertheless, for the Atlantic salmon used in this study, a raised incubation temperature appears to have a clear effect on muscle hyperplasia and hypertrophy with consequential negative effects on posthatch growth at least up to 3 weeks after the initiation of exogenous feeding.

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Diel vertical migration: A possible host-finding mechanism in salmon louse (*Lepeophtheirus salmonis*) copepodids?

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Abstract: The vertical distribution of pelagic nauplii and copepodids of the salmon louse *Lepeophtheirus salmonis* Krøyer was studied in large enclosures in the sea. Copepodids, which infect salmonid hosts, displayed a distinct diel vertical migration pattern. They gathered near the surface during the day, and spread out into deeper layers at night. Nauplii showed only small differences in depth between night and day. Copepodid distribution seems to be controlled by light intensity; no effect of either salinity or temperature was found. This migration pattern, which is the reverse of that of wild salmonids, may increase the number of parasite–host encounters as hosts will swim through populations of sinking (nighttime) and rising (dawn) parasites every 24 h. Because caged salmon feed at the surface during the day, they are likely to be more exposed to infective copepodids than wild fish.

Résumé : Nous avons étudié la distribution verticale des stades pélagiques nauplius et copépodite du pou du saumon *Lepeophtheirus salmonis* Krøyer dans de vastes enclos marins. Les copépodites, qui infectent les salmonidés, ont un cycle de migration verticale nycthémérale caractéristique : ils se rassemblent près de la surface pendant la journée et se répartissent dans les couches plus profondes pendant la nuit. Par contre, les nauplius varient peu leur profondeur entre le jour et la nuit. La distribution des copépodites semble dépendre de l'intensité lumineuse; la salinité et la température de l'eau ne semblent pas avoir d'effet. Cette migration, qui se déroule à l'inverse de celle des salmonidés sauvages, augmente peut-être la possibilité des rencontres entre le parasite et ses hôtes, ces derniers devant traverser les bancs de parasites qui descendent (la nuit) et remontent (le matin) toutes les 24 heures. Comme les saumons en cage se nourrissent à la surface pendant le jour, ils risquent d'être plus exposés que les saumons sauvages à l'infection par les copépodites.
[Traduit par la Rédaction]

Introduction

The salmon louse *Lepeophtheirus salmonis* Krøyer causes severe problems in both wild (Finstad 1992; Birkeland 1993; Tully et al. 1993) and farmed (Brandal et al. 1976; Pike 1989; Stuart 1990; Costello 1993) salmonids. This parasitic copepod feeds on skin and mucus, creating lesions, osmoregulatory problems, and secondary infections (Brandal et al. 1976; Wootten et al. 1982; Pike 1989; Johnson and Albright 1992; Jonsdottir et al. 1992). It has three planktonic developmental stages, nauplius I, nauplius II, and copepodid. The copepodid is the infective stage. On the salmonid host, the copepodid develops through four immobile chalimus stages and two mobile preadult stages before moulting to the mobile adult stage (Johnson and Albright 1991*a*; Schram 1993).

The behaviour of the copepodids probably enables them to stay in areas frequented by the host (Costello 1993). Laboratory experiments have shown the copepodid to be strongly phototactic (Johannessen 1978; Wootten et al. 1982; Bron et al. 1993), which suggests the presence of aggregations of larvae in surface layers during daylight. However, despite intensive sampling along coasts in several countries, pelagic swarms of copepodids have not been found (Irish Department of the Marine 1992, 1993). On the contrary, a few copepodids were found on the bottom of a bay in an Irish investigation (Irish Department of the Marine 1993). In this report it is hypothesized that the copepodids rest on the bottom during the day, and swim up into the water column at night when hosts are slow moving and surface oriented.

In the laboratory *L. salmonis* copepodids tend to cling to the sides of the containers, and frequently crawl out of the water (Johannessen 1978; Jakobsen and Holm 1990; personal observation). Based on these observations, the

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Table 1. Summary of conditions and procedures in the four experiments set up to investigate the vertical distribution of *Lepeophtheirus salmonis* larvae.

Parameter	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Sampling period (1993)	11–20 June	14–21 July	6–18 August	30 August – 7 September
Bag type	Black	Black	Black	Transparent
Bag depth (m)	6	6	6	12
Bag diameter (m)	2	2	2	0.65
No. of parallel bags	3	2	3	3
Surface temperature range (°C)	13.1–19.0	—	12.7–15.0	—
Average temperature below 1 m depth (°C)*	13.6±1.6	—	14.0±0.3	—
Surface salinity range (‰)	27.8–33.0	—	21.8–30.7	—
Average salinity below 2 m depth (below 1 m in exp. 3) (‰)*	32.0±0.8	—	30.4±0.4	—
Hatching period (days)	8	2	5	6
Sample size (L)	50 or 100	50 or 100	50 or 100	25

*Values are means ± SD.

hypothesis was advanced that copepodids attach to a substrate in the intertidal zone to save energy (Jakobsen and Holm 1990). To date there is no evidence to support this.

In the present paper we report a study of the vertical distribution of *L. salmonis* larvae in large enclosures in the sea, and describe a general pattern of diel migration.

Materials and methods

Experimental enclosures

Four experiments were performed at the Austevoll Aquaculture Research Station, Institute of Marine Research, near Bergen on the Norwegian west coast. Cylindrical black plastic bags, 2 m in diameter and 6 m deep, with slightly cone-shaped bottoms were used in experiments 1–3 (Table 1). The bags were suspended in the water from aluminium frames hung from a floating pier. In experiment 4, 12-m-deep transparent plastic bags with a diameter of 0.65 m, suspended in the water from circular frames were used as enclosures. The transparent bags were set up to check for artifactual distributions of larvae owing to the reduced amount of scattered light in the black bags. Bags in all four experiments were filled with sand-filtered seawater from 50 m depth, with a salinity of 30‰ and temperature of approximately 14°C. The black bags were replenished every two samplings, after temperature and salinity profiles were taken. Temperature and salinity readings were taken at 1-m depth intervals in all bags with a YSI model 33 S-C-T meter in experiment 1 and 3. Owing to technical difficulties, the temperature and salinity recordings in experiment 2 were incomplete. In experiment 4 the bags were refilled after each sampling, which produced a homogenous water column with respect to salinity and temperature. Details of temperature and salinity readings are given in Table 1. The salinity and temperature layers were broken down when the bags were replenished.

A separate experiment was conducted to determine if copepodids attach to a substrate. A 20 × 30 cm frame was wrapped in 180-μm white plankton mesh and half submerged in one of the black bags containing approximately 4000 copepodids. In the laboratory, copepodids readily attach to this mesh (Johannessen 1978; personal observation). The mesh was checked every 6 h for 3 days.

Animals and sampling

Egg strings were taken from *L. salmonis* females that were removed from salmon (*Salmo salar* L.) anaesthesized with metomidate (Mattson and Riple 1989). The fish were held in sea pens at the Austevoll Aquaculture Research Station, and had been naturally infected in the sea. Both pigmented and unpigmented strings were used. Maturation and hatching took place in 0.5-L plastic bottles with the bottom cut off and placed upside down. The bottles were filled with filtered (1.0-μm pore size) 30‰ salinity water from 50 m depth. Air entering through a tube in the cork provided circulation and oxygen. Up to 50 egg strings were placed in each bottle, and kept at 12°C with a 16-h light : 8-h dark cycle. Every morning the air supply was turned off, which allowed the hatched nauplii to gather at the surface. They were then decanted, and split into three (experiments 1, 3, and 4) or two (experiment 2) fractions containing roughly equal numbers of individuals. These were subsequently added to the experimental enclosures.

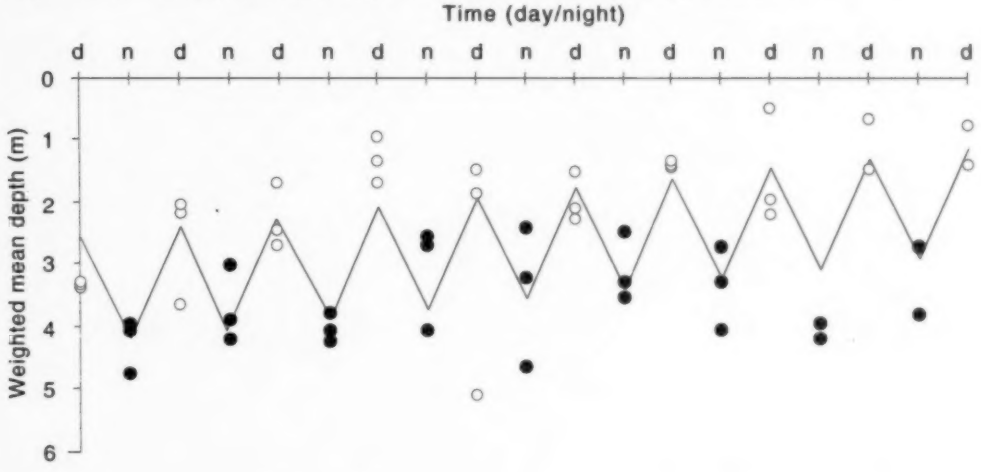
More than 10 000 nauplii were added to each bag, and sampling began 2–3 days after the last batch was put in to allow most animals to moult to the copepodid stage. In experiment 4, sampling started 1 day prior to the last day of stocking to ensure a maximum of nauplii in the bags. Duplicate 50- or 100-L samples were taken from the centre of the bags at 0, 2, 4, and 6 m in experiments 1–3. Duplicate 25-L samples were taken at 0, 2, 4, 6, 8, 10, and 12 m in experiment 4. The samples were taken with a Flygt type submersile impeller pump (ABS Coronado, 120 L/min with a hose length of either 8 m (experiment 1–3) or 15 m (experiment 4)). The pump housing consisted of a cylindrical case with diameter 20 cm and height 40 cm, and the inlets were slots around the sides of the bottom 3 cm of the case. For the deepest (bottom) samples the pump was lowered to the bottom and pulled up approximately 30 cm before it was started. Sampling, which took between 2.5 and

Table 2. Day and night weighted mean depths (WMD) of *Lepeophtheirus salmonis* in the four experiments. Differences were tested with Mann–Whitney *U*-tests at $\alpha = 0.1$.

Experiment No.	Stage	No. of days	No. of nights	Daytime WMD* (m)	Nighttime WMD* (m)	*U*	Difference night/day (Probability)
1.	Copepodid	10	9	1.95±0.21	3.61±0.22	120.33	<0.005
2.	Copepodid	8	8	2.49±0.26	3.63±0.26	100	<0.005
3.	Copepodid	4	4	1.98±0.36	3.29±0.36	15	<0.10
4.	Copepodid	3	3	2.82±0.92	6.63±0.92	9	0.10
4.	Nauplius	2	2	7.50±1.04	9.42±1.04		Not tested

*Values are means ± SE.

Fig. 1. Weighted mean depths (WMDs) of *Lepeophtheirus salmonis* for experiment 1, with line fitted according to a model consisting of two parallel lines. Day values are predicted by WMD = 2.57 − 0.16*t*, and night values by WMD = 3.42 − 0.16*t*, where *t* is time. Open circles are day WMDs, solid circles are night WMDs for three parallel bags. Bag 2 was terminated earlier than bags 1 and 3.



4 h to complete, was centered around the darkest time of night (01:35) and the brightest time of day (13:35). Samples were then filtered and the animals killed by adding freshwater. All samples were counted within less than 12 h after sampling. No attempt was made to distinguish between the nauplius stages. In some samples in experiment 4 there were animals moulting from nauplius II to copepodid that had parts of their abdomen covered with the old cuticle. The day samples from bag 1 and both day and night samples from bag 2 were examined for such larvae. Owing to a laboratory accident, the night samples from bag 1 on 30 August and the samples from bag 3 were not searched for moulting larvae.

Statistics

Weighted mean depths (WMDs) were calculated as WMD = $\Sigma n_i d_i / \Sigma n_i$, where n_i is abundance (mean of the duplicate samples/sample size) at depth d_i (e.g., as in Bollens and Frost 1991; Frost and Bollens 1992). Because n_0 is multiplied by $d_0 = 0$ and the $n_i d_i$s are summed, the smallest and largest WMD values possible equal the shallowest and deepest sample depth, respectively. Tested WMDs are means of WMDs from the three (two in experiment 2) parallel bags. Quartiles were calculated and plotted cumulatively according to Pennak (1943), and interquartile ranges (IQRs) extracted as the third to the first quartile. The proportions of copepodids at the surface were calculated as means of the duplicate samples from 0 m depth. Arcsine square root transformation of the proportions was carried out prior to the regression analyses. Statistics were calculated with the JMP statistical package (SAS Institute Inc., Cary, N.C.).

Results

Copepodids

The distributions of copepodids are presented as WMDs to discern the migration pattern, and as quartiles to show the spread. Generally, copepodids were found lower in the water at night than during the day. Night WMDs were significantly greater than day WMDs for both the black (experiments 1–3) and the transparent (experiment 4) type of bag (Table 2). The significance level was set to 0.1 owing to the small number of data points in experiments 3 and 4.

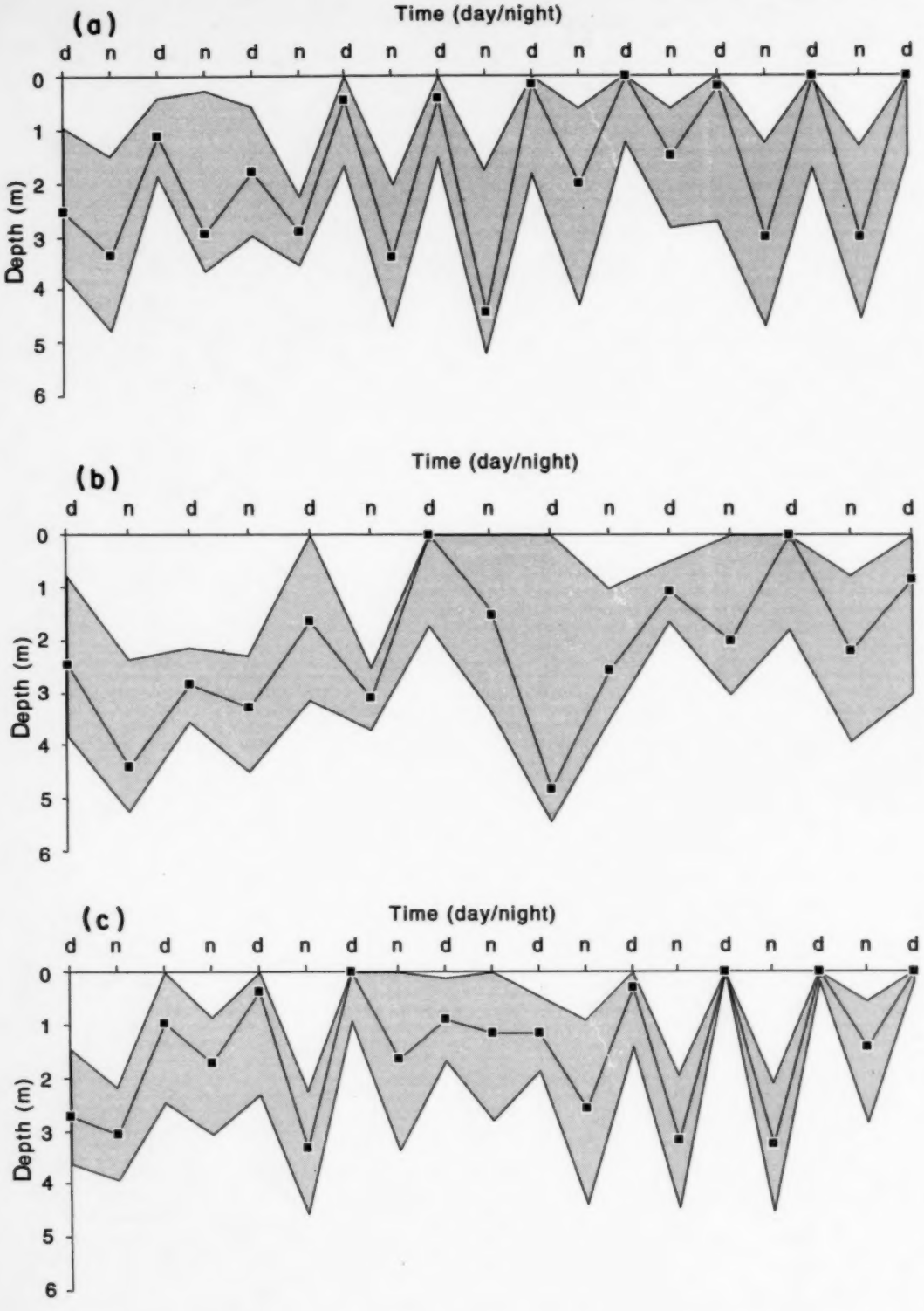

(a)
Time (day/night)
d n d n d n d n d n d n d n d n d n d
Depth (m)
0 1 2 3 4 5 6
(b)
Time (day/night)
d n d n d n d n d n d n d n d n d
Depth (m)
0 1 2 3 4 5 6
(c)
Time (day/night)
d n d n d n d n d n d n d n d n d n d
Depth (m)
0 1 2 3 4 5 6

Fig. 2. Experiment 1 quartiles for *Lepeophtheirus salmonis* plotted cumulatively according to Pennak (1943). Fifty percent of the sampled copepodids are in the shaded area. The middle line with filled squares is the median number of copepodids. Bag 2 (*b*) was terminated earlier than bags 1 (*a*) and 3 (*c*).

Experiment 1 was examined more closely as it had the most complete set of values for the approximately 10-day duration of the copepodid stage. A simple model including the variables night/day and time since the start of the experiment was fitted to the WMDs (Fig. 1). The model consists of two parallel lines. Day values are predicted by WMD = 2.57 − 0.16*t*, and night values by WMD = 3.42 − 0.16*t*, where *t* is time. The fit is significant, with $r^2 = 0.69$, and $p < 0.005$ for both factors (one outlier removed), indicating that the model can account for almost 70% of the variation in the data set. The residuals were found to be normally distributed by normal quantile plotting and the Shapiro–Wilk test. The model suggests that the mean amplitude of migration was 0.85 ± 0.09 m (mean ± SE) and did not change appreciably with time.

During experiment 1 there was a gradual rise of the copepodids in the water column over the experimental period (Fig. 1). This was apparently a linear change in depth, with a rise of 0.16 ± 0.03 m/day. Subsequent experiments did not confirm this trend, however.

The copepodids did not move up and down as a group. During the day there were high densities in the top 2 m, whereas the nighttime distribution was more spread out (Figs. 2*a*, 2*b*, and 2*c*). Spread, measured as interquartile range, was significantly larger in the nighttime samples in three of four experiments (Table 3) ($\alpha = 0.05$).

Nauplii

The distribution of nauplii was examined in experiment 4. Generally, nauplii were found deeper in the bags than copepodids. They seemed to stay higher in the water during the day than during the night (Table 2); however, the difference in WMDs was not tested owing to the small number of data points. The spread of the distribution measured as interquartile range was not significantly different between night and day (Table 3) at the 0.05 level of significance. There were, on average, four moulting nauplii per surface sample (0 m), between one and zero in the intermediate samples, and 12 in the bottom (12 m) samples ($n = 7$).

Distribution in relation to temperature and salinity

Surface temperatures (Table 1) were significantly different during day samplings as opposed to night samplings (*t*-test, $t_{0.05(2),44} = 2.834$, $p = 0.0069$ for experiment 1; $t_{0.05(2),18} = 5.586$, $p < 0.005$ for exp. 3). Linear regressions of this factor on the transformed proportions of copepodids in surface samples (P_0) showed that it could account for 1.3 ($p = 0.45$) and 16.6% ($p = 0.075$) of the variances, respectively. The difference in average temperature at 1-m depth between day and night was less than 1°C in both experiments.

The measured profiles indicated that salinity did not differ between night and day. Salinity profiles showed little change from 3 to 6 m depth on most sampling during experiment 1 (Table 1). All readings from this experiment were within the tolerance limit of *L. salmonis* copepodids (≥25‰; Johnson and Albright 1991*b*). Surface salinities ranged from 27.8 to 33.0‰, rising to between 31.0 and 33.5‰ at 3 m. There was no linear relationship between P_0 and surface salinity ($r^2 < 0.005$, $p = 0.91$). During experiment 3 the salinity was essentially uniform below 1 m depth, with an average of 30.39 ± 0.46‰ (mean ± SD). The 0-m salinities ranged from 21.8 to 30.7‰. The P_0s were not linearly related to surface salinity ($r^2 < 0.002$, $p = 0.86$).

The residuals of the model fitting of the values from experiment 1 did not indicate the influence of factors other than night/day and time (linear fit: $r^2 = 8 \times 10^{-19}$).

Attachment of copepodids

No copepodids were observed attached to the half-submerged frame with plankton mesh.

Discussion

General pattern of copepodid distribution

The copepodids of *L. salmonis* showed a distinct diel migration pattern. They gathered near the surface during the day, and spread out into deeper layers at night. The migration pattern was the same in the black and the transparent enclosures, indicating that the lack of scattered light from the sides in the black enclosures did not produce artifactual migrations.

Reproduction of natural vertical migration patterns in short laboratory water columns is a common phenomenon (Young and Watt 1993). As noted by these authors, zooplankton seem to scale their migrations to the available water depth. In the present work, this is seen as lower night WMDs in the deepest enclosures. The depth range of *L. salmonis* copepodid migration in the natural environment is unknown. In this discussion we relate our results to described taxes of salmon louse copepodids and other parasitic copepods, and to the migration of salmon and sea trout in estuarine areas.

In laboratory experiments, *L. salmonis* copepodids display positive phototaxis (Johannessen 1978; Wootten et al. 1982; Bron et al. 1993), as do many other caligid copepodids (e.g., *Lepeophtheirus dissimulatus* (Lewis 1963), *Caligus elongatus* (Hogans and Trudeau 1989; MacKinnon 1993; Pike et al. 1993) and *Caligus minimus* (Fraile 1989). It has been hypothesized that this serves to bring the infective copepodids to surface layers, where they may have a better chance of locating a suitable host (Wootten et al. 1982; Bron et al. 1993). No such aggregation has been found in nature, despite intensive searching (Irish Department of the Marine 1992, 1993). It is possible that light conditions in the cited laboratory studies were so different from natural underwater light that some of the described phototaxes are artifacts and do not indicate the distribution of the copepodids in the sea. Forward (1988) suggested that the use of direct light produces artifactual information about phototactic behaviour in many zooplankton species. The application of laboratory results to natural habitats should therefore be undertaken with caution.

In a study of the vertical distribution of salmon in pens, Huse and Holm (1993) found that the fish concentrated

Table 3. Estimated mean (±SE) interquartile ranges (IQR) for the day and night samples of *Lepeophtheirus salmonis* from the four experiments. Differences were tested with Mann–Whitney *U*-tests with normal approximation for experiments 1–3, and without normal approximation for experiment 4. Differences were judged significant at $\alpha = 0.05$.

Experiment No.	Stage	No. of daytime observations	No. of nighttime observations	IQR day samples (m)	IQR night samples (m)	*U* or *Z*	Difference night/day (Probability)
1.	Copepodid	28	25	1.86±0.17	2.72±0.18	3.5457	<0.005
2.	Copepodid	13	13	2.48±0.24	2.13±0.24	−1.0769	0.28
3.	Copepodid	12	12	1.90±0.26	2.79±0.26	2.1651	0.030
4.	Copepodid	7	7	2.20±0.92	6.17±0.92	42	<0.05
4.	Nauplius	6	6	6.53±1.36	3.27±1.36	26	>0.2

near the bottom of both 6 and 20-m-deep pens at high light intensities. Further, they found significantly more *L. salmonis* on the salmon from the 6-m-deep pen. This points to a greater abundance of copepodids in the upper 6 m of the water column than below.

The copepodids of *Lernaeenicus sprattae* (Sowerby) migrate upward at night to infect slow-moving shoals of sprat in surface layers (Schram and Anstensrud 1985). Contrary to *L. salmonis*, that can infect its host both at night (e.g., Johnson and Albright 1991*a*, 1991*b*) and during the day (Bron et al. 1991; personal observation), this parasite shows much reduced activity and infectivity at high light levels (Schram and Anstensrud 1985).

Several authors have reported that *L. salmonis* copepodids creep out of the water and cling to the substrate (Johannesen 1978; Jakobsen and Holm 1990; Boxaspen et al. 1993). This behaviour was also recorded for the parasitic copepod *Trebius caudatus* Krøyer by Sproston and Hartley (1941), who remarked that it is a feature of several other caligids as well. All the above observations were from laboratory work. No attached copepodids were found in the present investigation. It seems likely that the tendency to creep out of the water is a laboratory artifact. This is supported by the total absence of copepodids in samples from vegetation in the intertidal zone in Ireland (Irish Department of the Marine 1992).

Distribution of hosts

Our enclosure approach agrees with the hypothesis that copepodids gather close to the surface in daylight. If the patterns we observed are identical to natural copepodid behaviour, the question then becomes: how can this distribution maximize contacts with the salmonid hosts?

There is very little information on the vertical distribution of wild Atlantic salmonids in coastal waters. In Høgsfjorden, western Norway, Atlantic salmon (*Salmo salar*) smolts seem to travel in the brackish top 1–3 m of the water column, with short dives through the underlying halocline (Holm et al. 1982). Mature, homing salmon behave in much the same way. They follow layers in or just above density gradients such as the thermocline, with occasional deep dives (e.g., Westerberg 1982*b*; Døving et al. 1985). Dutil and Coutu (1988) state that Atlantic salmon postsmolts were most often caught in the upper 3 m of the water column, between 18:00 and 09:00. Reports on bycatches in different fishing nets indicate that during the night, nonmature salmon and sea trout frequent the 0- to 2-m layer more often than other layers in the 0- to 8-m range in fjords (Lund and Haukebø 1984). This behaviour has also been recorded for sea trout in an Irish sea lough (Minchin 1987).

Generally, caged salmon avoid the surface layer in daylight when not feeding there (Fernø et al. 1995; Juell et al. 1994), presumably to avoid predation. The fish descend in the morning and ascend in the evening (Fernø et al. 1995). This pattern is, however, modified by hunger level, season (Huse and Holm 1993; Fernø et al. 1995), and the presence of a thermocline (Fernø et al. 1995; Sutterlin and Stevens 1992). Hungry fish stay closer to the surface (Juell 1993), although this is balanced by light avoidance such that fish swim deeper in the summer than in the winter (Huse and Holm 1993; Fernø et al. 1995). Temperature may also affect swimming depth, as fish will occupy water layers with optimum temperatures for feeding and digestion (Fernø et al. 1995; Sutterlin and Stevens 1992). Both farmed and wild salmon and sea trout have periods of very little or no movement during the night (Westerberg 1982*a*, 1982*b*; Quinn et al. 1989; Furevik et al. 1993; Juell and Westerberg 1993).

Migration pattern of copepodids in relation to host distribution

It seems that both wild and farmed hosts stay deeper during the day than during the night, and farmed salmon at least rise to the surface layer in darkness. Our results indicate that salmon louse copepodids are high up in the water during daylight. However, the migration patterns would result in the hosts swimming through a population of sinking (nighttime) and rising (dawn) parasites every 24 h. According to Bron et al. (1993), *L. salmonis* copepodids probably respond to host-generated currents when settling, as do *L. pectoralis* (Boxshall 1976) and *C. minimus* (Fraile 1989). Migrating hosts will produce more and stronger currents, giving the copepodids more stimuli to direct them than passive, resting fish. This effects has been shown in laboratory experiments with the copepod *Salmincola edwardsii* Olson, that is parasitic on brook trout (*Salvelinus fontinalis*; Poulin et al. 1991). Finally, it is also possible that salmon lice are adapted to salmon with a large variance in their nighttime depths, or even to several salmonid host species having different behaviours at night. It might seem difficult for a drifting animal, as small as 0.7 mm, to attach to a swimming fish host of a much larger size. However, the

speed during swimming bursts is very high (Bron et al. 1993; personal observation). In front of the swimming fish pressure waves are formed (Kalmijn 1989) that may trigger a looping behaviour (Bron et al. 1993), thus making contact possible for the copepodids near the fish.

In typical fjords the upper layer of the water column is brackish, with layers of higher salinity underneath. Copepodids, which show reduced survival in 15‰ salinity (Johnson and Albright 1991*b*), may in such areas gather in the vicinity of the halocline during the day. Both homing salmon (Westerberg 1982*b*; Døving et al. 1985) and salmon smolts (Holm et al. 1982) swim close to and frequently through density gradients when they migrate in fjords. Thus, the described migration patterns for *L. salmonis* copepodids may also be advantageous in markedly stratified waters.

The present work indicates that copepodids do not rest on the bottom during the day and swim up in the water column at night. The epibenthic copepodids found in the Irish study (Irish Department of the Marine 1993) may have been in the very first part of, or very near the end of, their pelagic lives. In our experiments, small numbers of inactive larvae were quite frequently found in bottom samples.

During experiment 1 the copepodids occupied progressively higher positions in the water column. It is possible that copepodid physiology changes during their 10-day pelagic life, such that sensitivity to light is adjusted. A change in buoyancy may also be implicated. The result was not confirmed by later experiments, possibly because of the smaller number of samples taken, and varying weather conditions during the experiments.

Distribution of nauplii

The nauplii of *L. salmonis* are reported to be less active and less responsive to light than copepodids (Johannessen 1978; Wootten et al. 1982; Bron et al. 1993). This is consistent with the results of the present investigation, which showed lower WMDs and a smaller difference between night and day depths for nauplii. Johannessen (1978) stated that moulting nauplii II remained near the bottom of laboratory beakers, and showed little swimming activity. It was, therefore, surprising to find moulting nauplii at the surface in our 12-m-deep bags. A possible reason for this might be that living conditions were better in the bags than in laboratory beakers, or that it was a consequence of having much larger numbers of larvae in the experiment.

Distribution of copepodids in relation to temperature and salinity

The temperatures and salinities recorded did not seem to influence the diel migration pattern of the copepodids. Although the surface temperature was lower at night, it could not explain the diel change in the proportion of copepodids in the surface samples. This is in accordance with current views on the timing of zooplankton migration (Lampert 1988). The lowest salinity reading at the surface was 21.8‰, which should be at the tolerance limit of *L. salmonis* copepodids (Johnson and Albright 1991*b*). A general avoidance of the lower salinities was, however, not found. This may be due to the fact that the pump collects a sphere of water. A thin low-salinity layer at the surface with few copepodids would not have been detected if high densities of copepodids were at the halocline immediately underneath. The influence of pronounced halo- or thermo-clines on the vertical migration remains to be investigated.

Our results strongly suggest that light intensity is of major importance for the vertical distribution of salmon louse copepodids. Their diel migration pattern may have consequences for fish farming. Whereas wild fish have enough light and space to forage well away from the surface in daylight, farmed fish eat at the surface several times a day. Consequently, it may be that the presence of copepodids gathering in surface layers when the fish are fed is one of the reasons for the high lice loads on domesticated salmonids.

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Field transplantation of a freshwater bivalve, *Pyganodon grandis*, across a metal contamination gradient. I. Temporal changes in metallothionein and metal (Cd, Cu, and Zn) concentrations in soft tissues

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Abstract: To test the response of the freshwater bivalve *Pyganodon grandis* (formerly *Anodonta grandis*) to increased metal exposure in the field, we transferred specimens (8 cm length; 4–6 years old) from a less to a more contaminated lake in the mining area of Rouyn-Noranda, in northwestern Québec. The transplanted bivalves were maintained in open enclosures placed in the bottom sediments of the contaminated lake. Up to 16 individuals were removed from pairs of enclosures at times $t = 0$ (June 1990), 5, 14, 30, 60, 90, and 400 d; tissue concentrations of metallothionein (MT) and metals were monitored over time. Measurements on control molluscs enclosed in their lake of origin showed that enclosure per se had no apparent effect on tissue [MT] or tissue metal levels, but did decrease shell growth. Metallothionein levels in specimens transplanted to the more contaminated lake showed a slow but steady increase with time; in contrast, MT levels in the control populations showed only modest seasonal fluctuations. The increase in MT over time in the transplanted bivalves was closely correlated with a similar slow increase in soft tissue [Cd]. We conclude that MT in the freshwater bivalve *P. grandis* is a promising biochemical indicator of metal exposure.

Résumé : Pour évaluer la réponse du bivalve *Pyganodon grandis* (anciennement *Anodonta grandis*) à une brusque augmentation de métaux en milieu naturel, nous avons transplanté des spécimens de ce bivalve (longueur 8 cm; 4–6 ans) d'un lac relativement peu contaminé vers un lac très contaminé dans la région minière de Rouyn-Noranda, dans le nord-ouest du Québec. Les bivalves transplantés et témoins (gardés dans leur lac d'origine) furent confinés dans des enclos enfoncés dans les sédiments littoraux. Jusqu'à 16 individus furent retirés de chaque série d'enclos aux temps 0 (juin 1990), 5, 14, 30, 60, 90 et 400 jours; les concentrations de métallothionéine (MT) et de métaux furent suivis dans le temps. Le confinement des mollusques n'affecta pas les niveaux tissulaires de MT ou de métaux, mais provoqua un ralentissement de la croissance en longueur de la coquille. Les bivalves transplantés vers le lac le plus contaminé montrèrent un accroissement lent de MT dans le temps; cet accroissement était corrélé avec une augmentation tout aussi lente des concentrations tissulaires de Cd. Les variations saisonnières de [MT] dans les bivalves témoins étaient modestes en comparaison avec la hausse régulière en [MT] notée chez les bivalves transplantés. La métallothionéine dans le bivalve d'eau douce *P. grandis* s'avère prometteur comme indicateur biochimique d'exposition aux métaux.

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Introduction

Traditionally, attempts to assess the impacts of contaminants on aquatic ecosystems have involved laboratory experiments performed under defined conditions (toxicity tests) and, to a lesser extent, field observations on impacted indigenous populations. To date these approaches have met with only limited success (NRCC 1985; Cairns 1992). Extrapolation of laboratory-derived toxicological data to the field is fraught with difficulties, as is the unambiguous interpretation of field observations, e.g., changes in relative abundance, species diversity, community structure, and biomass. An alternative and complementary approach involves the use of biochemical indicators to monitor the response of individual organisms to toxic chemicals.

According to the biochemical indicator concept (NRCC 1985), behavioral effects at the organism level and those operating at the population, community, and ecosystem levels are preceded by biochemical reactions in individual organisms. Concentrations of a contaminant needed to initiate these reactions are assumed to be lower than those required to provoke a life-threatening situation for the target organism or perceptible degradation of the ecosystem. The detection and quantification of these biochemical reactions could then be developed as an early, sensitive, and specific indicator of environmental stress (NRCC 1985). The following criteria for such indicators have recently been proposed (Stegeman et al. 1992; Haux and Förlin 1989).

(1) The indicator should present an early warning capacity, i.e., the biochemical response should be predictive of effects at higher levels of biological organization (population, community) and should precede them.
(2) It should be specific to a particular contaminant or a class of contaminants.
(3) It should respond in a concentration-dependent manner to changes in ambient levels of the contaminant.
(4) Endogenous and exogenous factors that affect the indicator should be known, so that sources of uncontrolled variation can be minimized.
(5) The indicator should be related to the health or fitness status of the organism.

For metals, much of the attention regarding biochemical indicators has focused on metal-binding proteins. Metallothioneins (MT) are low molecular weight, cysteine-rich metal-binding proteins that are ubiquitous in the animal kingdom and show high affinity for Group IB and IIB metal ions. Studies involving aquatic animals have suggested a central role for these molecules in the regulation of the essential metals Zn and Cu; in the detoxification of these metals, when present in excess, and of the nonessential metals Cd and Hg; and in the acquisition of metal tolerance for populations living in metal-contaminated environments (Roesijadi 1992). The vast majority of studies supporting this model have, however, been performed in the laboratory at artificially high metal concentrations and for short exposure times. Extrapolation of this model of MT involvement in metal metabolism to field conditions is accordingly tenuous. More has to be determined about the physiological and toxicological functions of MT in organisms in their natural environment (Roesijadi 1992; Stegeman et al. 1992; Luoma and Carter 1991; Viarengo 1989) before definitive conclusions can be drawn as to the usefulness of MT as a biochemical indicator.

Earlier work in our laboratory demonstrated the presence of a MT-like protein in the gills and the digestive gland of specimens of the freshwater bivalve *Pyganodon grandis* (formerly *Anodonta grandis*) that had previously been exposed to artificially high concentrations of Cd in the laboratory. This metal-binding protein was subsequently detected in specimens collected from lakes in a mining area (Couillard et al. 1993; Legrand et al. 1987). In the present paper we evaluate the potential of MT in *P. grandis* as a biochemical indicator of metal exposure. Specimens of this bivalve were transplanted from a relatively unpolluted lake to a lake contaminated by Cd and Zn. Our objectives were to assess the seasonal variability in MT levels of specimens kept in their source lake (criterion 4), and to compare this variability with that caused by an abrupt increase in ambient metal levels (criterion 3). Provided relatively stable conditions prevail over time in the contaminated lake, MT and metal concentrations in transplanted mussels would be expected eventually to reach those in the indigenous bivalves. A companion paper (Couillard et al. 1995) extends this evaluation of MT and examines its conformity to criterion 1 (early warning capacity) and criterion 5 (links to organism fitness).

Materials and methods

Study area

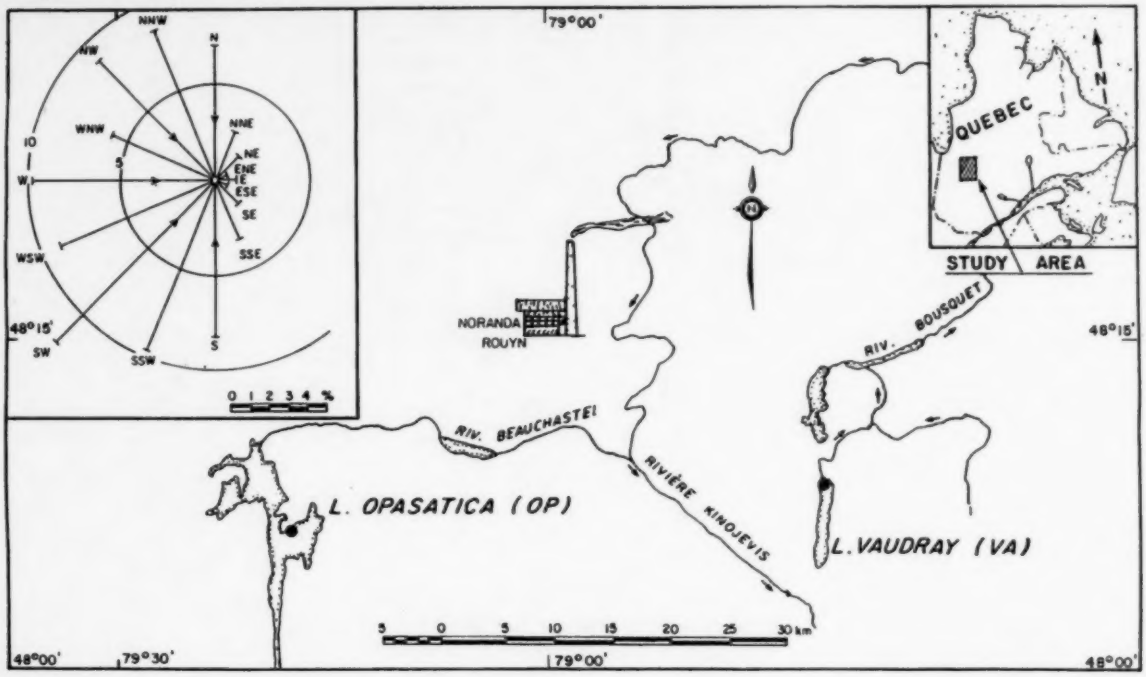
Since 1927, major mining and smelting operations have been carried out in the Rouyn-Noranda region in northwestern Québec. According to data available for 1977, annual atmospheric emissions from the smelting complex included 485 000 tonnes (t) SO_2, 75 t Cd, 34 t Cu, 1540 t Pb, 610 t Zn, and 16 t Hg. Lesser emissions of Bi, Co, Fe, Ni, Sb, Se, and Te were also reported (BEST 1979*a*). Sediment cores obtained in 1982 indicated that surficial sediments in lakes located within 10 km of the smelters were severely contaminated with Cu, Zn, and Pb; sediment enrichment factors ($[M]_{0\text{–}7\ cm}/[M]_{background}$, where M = metal) ranged from 118 for Zn to 166 for Cu (Arafat 1985). Air and water pollution controls have recently been introduced, but surficial sediments in lakes surrounding the smelter still reflect their history of contamination.

In a study carried out in the region in 1989, we demonstrated that MT concentrations in indigenous populations of *P. grandis* were significantly correlated with the free Cd^{2+} concentration at the sediment–water interface, as estimated from sediment–water sorptive equilibria (Couillard et al. 1993). As the present experiment was designed to monitor changes resulting from increased metal exposure as a function of time, we selected two lakes from the 11 lakes sampled in the former study (lakes Opasatica and Vaudray; Fig. 1). These two lakes were of similar trophic status but of widely different levels of Cd and Zn contamination; metallothionein concentrations in the bivalves also differed markedly between the two lakes (Table 1). The rationale for choosing these lakes is discussed below.

Experimental design

Specimens of the benthic bivalve *P. grandis* of a nominal length ≈8 cm were transplanted from Lake Opasatica (low

Fig. 1. Location of the study sites, indicated by solid circles. Average wind directions at the Rouyn-Noranda smelting complex are shown in the left-hand inset.



metal contamination) to Lake Vaudray (high metal contamination) (N = 112; mean length = 80.1 ± 4.3 mm (mean ± SD); herein referred to as T-VA). Indigenous mussels from both Lake Opasatica (referred to as C-OP: N = 64; length = 81.3 ± 4.5 mm) and Lake Vaudray (referred to as C-VA: N = 112; length = 82.6 ± 3.9 mm) served as control groups. None of the animals were found to be gravid in a preliminary sampling carried out 2 weeks before the transplantation experiment. All groups, including the controls, were maintained within enclosures (plastic borders, 10 cm high, arranged in circles of 60 cm diameter) inserted into littoral sediments (water depth ≈5 m). The enclosures, 24 in Lake Vaudray and 6 in Lake Opasatica, were arranged in pairs along a rope anchored on the lake bottom. Each enclosure contained eight molluscs; the resulting density of 8 animals/0.28 m^2 was comparable with that of the indigenous populations. The animals transferred to Lake Vaudray (T-VA) and the Lake Vaudray controls (C-VA) were sacrificed according to the sequence 0, 5, 14, 30, 60, 90, and 400 d (t = 0: 20 June 1990; two enclosures sampled and thus 2 × 8 = 16 molluscs collected on each date, unless mortality had occurred). The C-OP control group was sampled at times 0, 5, 90, and 400 d. In coding the various groups, we have used the following conventions: C = control; T = transferred; OP = Lake Opasatica; VA = Lake Vaudray; IND = indigenous, free living. The number of days of exposure completes the code, e.g., T-VA-90 d designates molluscs transferred to Lake Vaudray and collected after 90 d exposure.

To follow the growth of individual organisms, we identified specimens destined to be collected at 400 d by engraving a number with a sharp nail on the umbone region of the shell. Similarly, to determine whether marking the bivalves and maintaining them within the enclosure had an influence on metallothionein and metal concentrations, and on condition indices, we collected indigenous bivalves on day 400 outside the enclosures in each lake (samples referred to as OP-IND and VA-IND) and compared them to marked specimens from groups C-OP-400 d and C-VA-400 d. For the groups C-OP-400 d and C-VA-400 d, we also evaluated the possibility of enclosure and marking effects on shell growth 90 d after initiating the experiment; this evaluation will be discussed later.

Bivalve ageing

To compare the ecological characteristics of the contaminated and control sites, we assessed the growth rates of their indigenous bivalve populations. Two weeks before the transplantation experiment, we selected 21 and 29 specimens, respectively, from lakes Opasatica and Vaudray; specimens were chosen to represent the range of size classes present. The external annual growth rings were then counted independently by two evaluators. Differences between the independent age estimates were infrequent (5 differences among 50 determinations). Shell lengths as well as interannular lengths were measured to the nearest 0.1 mm using digital calipers. Shell length is defined as the longest dimension of the shell, measured with a caliper without compressing the outer shell margin. Interannular length is the widest dimension within a given annual ring, i.e., along the axis of greatest shell length. The age of each of the

Table 1. Surface areas, geochemical and biological data for the two sites chosen for the transplantation experiment.

	Geochemical data			Biological data	
	Lake Opasatica	Lake Vaudray		Lake Opasatica	Lake Vaudray
			Whole organism		
Area (ha)	5128	746	[MT] (nmol M sites·g^{-1})	154±10	596±16
[Ca] (10^{-4} M)	1.65	0.9	[Cd] (nmol·g^{-1})	178±6	1560±60
Gran alkalinity (meq·L^{-1})	292	93	[Cu] (nmol·g^{-1})	680±43	1260±60
DOC (mg C·L^{-1})	6.5	6.5	[Zn] (nmol·g^{-1})	3130±150	4880±280
Mean pH[a]	7.39	6.56			
Dissolved [M]			Gills		
[Cd] (nM)[b]	0.8	1.2	[MT] (nmol M sites·g^{-1})	109±14	411±20
[Cu] (nM)[b]	56	53	[Cd] (nmol·g)	279±19	2560±150
[Zn] (nM)[b]	40	174	[Cu] (nmol·g)	1435±55	3060±200
Calculated [Cd^{2+}] (nM)[c]	0.28	2.2	[Zn] (nmol·g)	6660±260	10 140±830
[M] in oxic sediments			Organism characteristics		
Extract. [Cu] (nmol·g^{-1})[d]	54	88	Condition index	0.110±0.003	0.103±0.009[e]
Extract. [Zn] (nmol·g^{-1})[d]	150	1200			
Total [Cd] (nmol·g^{-1})	4.4	38	Growth rate	Similar[f]	
Total [Hg] (nmol·g^{-1})	0.10	0.10			

Note: Geochemical data are from Couillard et al. (1993). Biological data were determined on specimens collected outside the enclosures at the end of the experiment (OP-IND and VA-IND: $N = 3$ replicates; each replicate comprises four animals; mean ± SE). Metal (M) and MT concentrations in sediments and biological tissues are expressed on a dry weight basis.

[a]Mean pH estimated from summer time series (June to October); mean pH = $-\log_{10}(\Sigma[H^+]/N)$.

[b]Single measure at 10 cm above the sediments (June 1989).

[c]$[Cd^{2+}]$ estimated from sediment/water sorptive equilibria; see Tessier et al. (1993) for details.

[d][M] extracted for 6 h at 96°C with 0.04 M NH_2OH·HCl in 25% (vol/vol) HOAc.

[e]A condition index based on soft tissue dry wt. standardized by intervalval volume gave similar values for the two sites. See Figure 5 in Couillard et al. (1993).

[f]See Figure 2*a*.

specimens making up the groups C-OP-400 d and C-VA-400 d was also determined.

Comparison of ecological characteristics of the two sites depended on accurate age and interannular length determinations. In a few cases, shell erosion made it difficult to distinguish the first annulus. In the determinations of age–growth relationships for the indigenous bivalves, we avoided trying to discriminate among crowded rings on the shell edges of older specimens. Such concerns were raised by Metcalfe-Smith and Green (1992) with reference to the use of external rings of unionids for ageing. The appearance, once a year, of a new external growth ring for populations of bivalves from northern climates has been generally accepted, based on mark–recapture studies (reported by Metcalfe-Smith and Green 1992; *P. grandis*, Ghent et al. 1978). However, in a recent experiment of this type, Downing et al. (1992) observed that external annuli on several shells of *P. grandis* from a lake in Minnesota were formed less frequently than annually. The generality of this observation cannot be established at this time.

Bivalve analyses

The bivalves were dissected within 4 h of collection into four tissue groups: gills, mantle, digestive gland, and remaining tissues (foot, visceral mass, labial palps, kidneys, heart and muscle, which are hereafter referred to as the remainder). To minimize the influence of ingested particulate material on the estimation of metal concentrations in the remainder (Hare et al. 1989), the animals' gut contents were removed during the dissection by flushing the digestive tracts with deionized water. Tissues from four animals were pooled yielding four replicates per treatment and per date when no mortality had occurred. Tissues were put in two polyethylene bags: the inner bag was vacuum sealed, and the outer was filled with nitrogen. They were stored at −40°C until homogenization, carried out 1–5 months later.

Partially thawed tissues were homogenized with a tissue grinder (Brinkman Kinematica CH-6010) in ice-cold 25 mM Tris solution adjusted to pH 7.2. Weights of added buffer solution varied for the different tissues: digestive gland (4:1 wt:wt ratio of buffer:tissue), mantle (3:1), gills (2:1), or remainder (1:1). Homogenization was performed in a glove bag filled with nitrogen (Atmosbag, Aldrich Chemical Co.) to minimize oxidation of MT during this step, and the homogenized tissues were kept on ice. A subsample (3 mL) was centrifuged at 30 000 × *g* for 30 min at 4°C and the supernatant was analyzed the same day for MT. Additional subsamples of the homogenate were retained for determinations of Ca, Cd, Cu, and Zn concentrations. To determine the dry weight (wt.) to wet wt. ratio, an additional

subsample of the tissue homogenate was weighed before and after drying at 70°C for 24 h; dry wt. to wet wt. ratios were corrected for the contribution of the added buffer. The following condition index (CI) was calculated for each of the replicates (Lucas and Beninger 1985):

$$[1] \quad CI = \frac{\text{total flesh dry weight (g)}}{\text{total shell weight (g)}}$$

Metallothionein

Metallothionein concentrations in the various tissues were measured with a mercury saturation assay adapted slightly from Dutton et al. (1993) and described in detail in Couillard et al. (1993). The recoveries of MT standards during the assay (MT from rabbit liver, Sigma Co.; stoichiometry of 7 mole metal·mole^{-1} MT) were in the ranges of 87–114, 85–110, 85–114, and 87–113% for the gills, mantle, digestive gland, and remainder, respectively.

Metallothionein concentrations are expressed as nanomoles metal binding sites per gram of dry tissue wt. (based on measured Hg binding capacities). Concentrations in the whole organism, [MT(org)], were calculated as follows:

$$[2] \quad [MT(org)] = \frac{\sum([MT(tissue)]_i W_i)}{\sum W_i}$$

where $[MT\ (tissue)]_i$ and W_i are the metallothionein concentration and the dry wt. of the ith tissue, respectively.

Tissue metal analyses

An amount of tissue homogenate corresponding to 100 mg dry wt. was pipetted into a 30-mL Teflon bomb (Parr Instrument Co.) and preheated for 2 h in an oven at 70°C; ultrapure concentrated nitric acid (3 mL; BDH Chemicals, Aristar grade) was then added and the digestion carried out in a microwave oven (700 watts, ≤2 min) up to a pressure of 6900 kPa. Cooled digested samples were diluted with deionized water up to a volume of 25 mL and Cd, Cu, and Zn concentrations were determined by plasma atomic emission spectrometry (Thermo Jarrell Ash, Atom Scan 25; sequential reading). Samples of similar weight of a certified reference material (U.S. National Institute of Standards and Technology, NIST; oyster tissue, SRM No. 1566) were digested during every other analytical run; measured trace metal concentrations in the standard varied little over time (coefficients of variation 3–17%, $N = 14$) and were within the certified ranges for each element. Low to negligible contamination was detected in digestion blanks (Cd, 0.03 ± 0.03 nmol·g^{-1} dry wt.; Cu, 5 ± 2.1 nmol·g^{-1} dry wt; Zn, 7.6 ± 5.1 nmol·g^{-1} dry wt.; Ca, 89 ± 25 nmol·g^{-1} dry wt.; $N = 12$; calculated for a nominal tissue weight of 100 mg).

Metal concentrations in the whole organism, [M(org)], were calculated as described above for metallothionein (Eq. 2).

Labware

To minimize trace element contamination, all labware was soaked in 15% nitric acid for 24 h and rinsed repeatedly in deionized water prior to use. High purity water for analytical purposes (>17 Mohms/cm) was obtained from a commercial system with mixed-bed ion exchange, charcoal adsorption, and filtration (0.2 μm) steps.

Statistical analyses

One-way analyses of variance were applied to test for significant differences in the temporal profiles of MT, metals, and other measured variables for each treatment series: T-VA, C-OP, and C-VA. Differences among successive group means (T-VA-i d, T-VA-j d, etc.) were examined using a posteriori Scheffe contrasts. Because the groups in each treatment had unequal sample sizes (owing to mortality), a priori orthogonal contrasts could not be used. Increases in MT or metal concentrations in the transplanted bivalves were considered significant when the T-VA replicates were significantly different from the C-OP replicates collected after the same exposure time (Student t-test for differences between two means) and were significantly different from the T-VA replicates collected at $t = 0$ d. Relationships between MT and tissue metal concentrations were initially examined in bivariate scatterplots and tested by calculating Pearson correlation coefficients (r). Partial correlation analyses were used to detect spurious relationships between MT and tissue metal concentrations, notably those caused by changes in organism dry weights. Shell growth rates of the bivalve populations from the two lakes studied were determined using bivariate plots of the length delimited by an annulus in year $n + 1$ versus length for year n, the so-called Walford plots (McCuaig and Green 1983). The similarity between the two linear regressions was tested by an analysis of covariance (ANCOVA). The assumptions required for use of these parametric methods were generally met with the raw (nontransformed) data.

Results and discussion

Comparison of the two transplantation sites

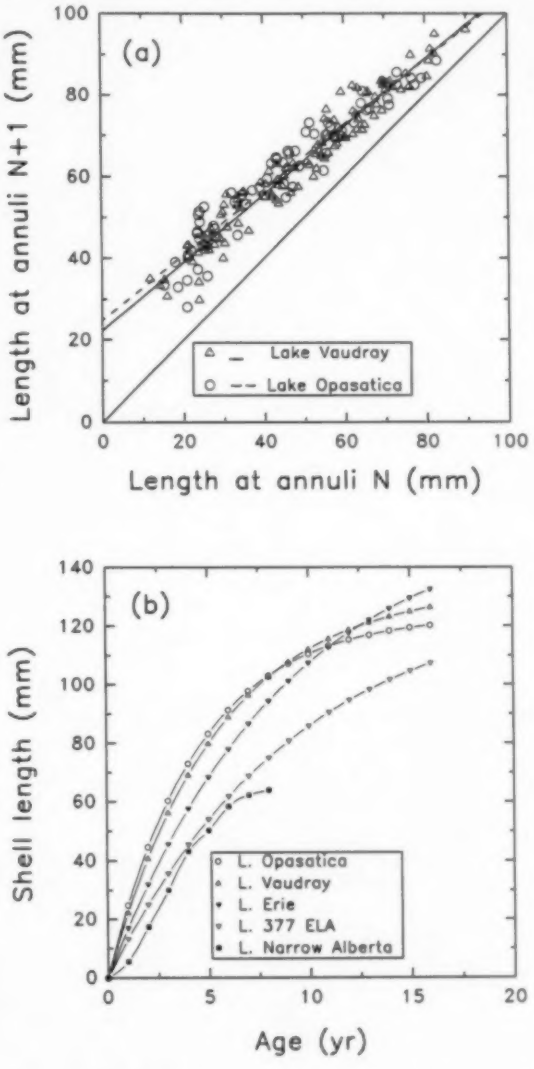
Lake Opasatica is situated upwind from the Rouyn-Noranda smelting complex whereas Lake Vaudray is located downwind (Fig. 1). Both lakes lie at elevations of less than 300 m on glaciolacustrine deposits left by the postglacial Lake Barlow-Ojibway (Surficial geology map No. 1639A, Geological Survey of Canada; Energy, Mines and Resources Canada 1987). Superimposed on this geological similarity, the chosen sites exhibit the desired differences in the levels of contamination of their biotic and abiotic components, as shown in Table 1. The trace metal concentration differences are greatest for Cd and Zn in the surficial oxic sediments, and for estimated dissolved Cd^{2+} (eight- to nine-fold differences). H^+ ion activities, Gran alkalinity values and dissolved Zn also show appreciable differences between the two sites, whereas the remaining variables, notably [Ca], [DOC], dissolved [Cu], and sediment [Cu], are of similar magnitude. The two sites are apparently not contaminated by Hg, also a potential inducer of MT biosynthesis. Mercury concentrations in the surficial sediments fall within the range of sediment background levels for the region, 0.10–0.45 nmol Hg·g^{-1} dry wt., as determined in pre-1900 strata from lake sediment cores (BEST 1979*b*).

Metallothionein and Cd concentrations in the tissues of *P. grandis* are considerably higher in indigenous specimens from Lake Vaudray than in those from Lake Opasatica; tissue Cu and Zn levels also differ between the two sites but not to the same extent as the Cd concentrations (Table 1). Whereas the tissue concentrations of the essential elements

(Cu and Zn) are not unusual for freshwater lamellibranch molluscs (cf. Manly and George 1977: 340–1630 nmol $Cu \cdot g^{-1}$ dry wt., 6170 – 27 800 nmol $Zn \cdot g^{-1}$ dry wt.; whole body concentrations), MT and Cd levels are much higher than those reported for indigenous populations from uncontaminated systems. For example, for specimens of *P. grandis* living in a pristine Precambrian Shield lake in northwestern Ontario, Canada (0.01 ± 0.001 nM dissolved Cd), Malley et al. (1989) reported a mean whole body concentration of ≈36 nmol $Cd \cdot g^{-1}$ dry wt. and a gill MT concentration of ≈9 nmol metal binding $sites \cdot g^{-1}$ wet wt. (estimated from Malley et al. 1993, assuming a molecular weight of 10 kDa for MT and a stoichiometric ratio of 7 mole $Hg \cdot mole^{1}$ MT). On a wet wt. basis, gill MT concentrations for the relatively uncontaminated bivalve population of Lake Opasatica were somewhat higher (16 ± 2.6 nmol metal binding $sites \cdot g^{-1}$ wet wt.).

To minimize the influence of factors other than metal contamination on the biochemical and physiological responses considered, we selected sites likely to share similar ecological characteristics. It is known that environmental conditions and, in particular, quality and availability of food are well integrated by the condition index (Lucas and Beninger 1985) and shell growth rate (McCuaig and Green 1983) of bivalve populations. Comparison of these characteristics for the indigenous mollusc populations suggested that the two sites were indeed of similar trophic status (Table 1 and Fig. 2a). For example, the length delimited by an annulus for year $n + 1$ was regressed against the length for year n for samples of mussels collected at the sites in lakes Opasatica and Vaudray 2 weeks before the beginning of the experiment. The regression coefficients and the Y-intercepts are not significantly different (ANCOVA: $F_{slope}(1, 174) = 1.13$; $F_{position}(1, 175) = 2.48$; $P > 0.05$). The regressions in Fig. 2*a* were used to derive the Von Bertalanffy shell growth curves (McCuaig and Green 1983; Fig. 2*b*). Visual comparison of these shell growth curves again suggests that the growth rates for the bivalves are similar in lakes Opasatica and Vaudray. In addition, these bivalves apparently grow faster and to larger sizes than bivalve populations at similar latitudes in Canada, e.g., in Lake 377, Experimental Lakes Area, Ontario (Malley et al. 1989) and at a 1-m depth in Lake Narrow, Alberta (Hanson et al. 1988). However, they are apparently surpassed in asymptotic length by *P. grandis* from Lake Erie (McCuaig and Green 1983). If shell growth rate is to be assessed for the purposes of the transplantation experiment, then water turbulence must be taken into consideration because of its effect on the allometric growth of the shell (Green et al. 1989). Because both sites were located at similar depths and close to the lee shore (Fig. 1), it seems reasonable to assume that the bivalves experienced similar water turbulence regimes. We conclude that the two transplantation sites are ecologically similar.

Fig. 2. (*a*) Walford plots of lengths at consecutive annual rings for the bivalve *P. grandis* from lakes Opasatica ($N = 21$) and Vaudray ($N = 29$). The specimens were collected ? weeks before the transplantation experiment. The solid line originating from 0,0 is the line of zero growth. (*b*) Von Bertalanffy growth curves for populations of *P. grandis* from some lakes in Canada. Data source: lakes Opasatica and Vaudray, this study; Lake Erie, McCuaig and Green (1983); Lake 377 ELA, Malley et al. (1989); Lake Narrow, Hanson et al. (1988).



Evaluation of an enclosure and marking effect on the bivalves used in the transplantation experiment (control groups C-OP and C-VA)

Before discussing the effects of the changed metal exposure regime on tissue MT and tissue metal concentrations, we should consider whether these latter parameters were affected by the experimental design (i.e., by enclosing the molluscs and restricting their movement). For enclosed specimens in Lake Opasatica (treatment C-OP), comparisons between marked mussels in the enclosures of the group C-OP-400 d and indigenous mussels collected outside the enclosures at the end of the experiment (OP-IND) did not reveal any corral or marking effect on condition index (Fig. 3), on MT, nor

Fig. 3. Variations over time of condition indices of transplanted and control bivalves. T-VA = transplanted bivalves; C-OP and C-VA = specimens put in enclosures in their source lakes Opasatica and Vaudray, respectively; OP-IND and VA-IND = specimens collected outside the enclosures in lakes Opasatica and Vaudray, respectively.

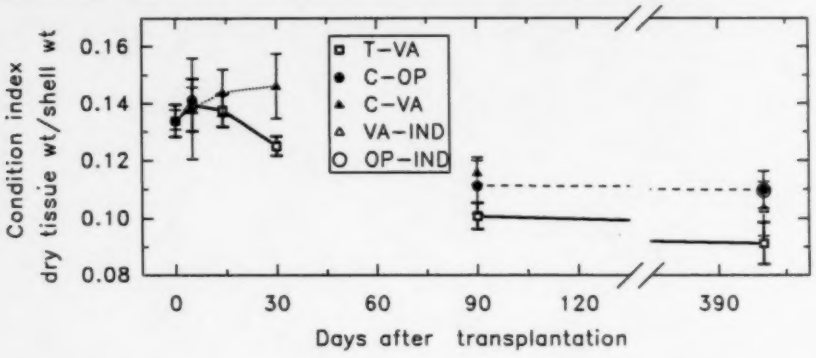


Table 2. Comparisons between marked bivalves kept in enclosures (group C-OP-400 d) and their congeners collected outside the enclosures at the end of the experiment (OP-IND).

Group identification	Enclosure and marking effect on			
	[MT]	[Cd]	[Cu]	[Zn]
Gills				
C-OP-400 d	No	No	No	No
Mantle				
C-OP-400 d	Yes ($P \approx 0.02$)	No	No	No
Digestive gland				
C-OP-400 d	No	No	No	Yes ($P \approx 0.04$)
Remainder				
C-OP-400 d	No	No	No	No
Whole organism				
C-OP-400 d	No	No	No	No

Note: Replicate sample sizes: groups C-OP-400 d and OP-IND, $N = 3$.

on tissue metal concentrations (with the exception of [MT(mantle)] and [Zn(digestive gland)]: Table 2). The marking effect concerns only the enclosed specimens collected at 400 d; specimens destined to be sacrificed earlier in the experiment were not marked as individual growth measurements were not planned for these animals.

Organism dry weights decreased over time in all treatments, C-OP, C-VA, and T-VA, affecting the mollusc condition indices (Fig. 3). A similar decrease also occurred in the indigenous free populations in the two lakes (Fig. 3; compare C-VA-0 with VA-IND, C-OP-0 with OP-IND). Cain and Luoma (1990) also observed a constant decrease for 2 consecutive years in soft tissue weight in indigenous specimens of the marine bivalve *Macoma balthica* in San Francisco Bay. Yearly profiles (with a high sampling frequency) for several consecutive years would be needed to evaluate interannual variations in condition index and to relate these to year-to-year variations in such factors as the abundance and nutritional quality of the suspended particulate matter.

We were unable to perform similar comparisons between the marked mussels in the C-VA-400 d group and the indigenous mussels collected in Lake Vaudray at the end of the experiment (VA-IND). Mortality was much higher for the group C-VA-400 d than for the other groups (Table 3); numbers of live bivalves among the 16 marked individuals after 90 and 400 d were, respectively, 7 and 1. In comparison, at the end of the experiment we recovered 14 live individuals from the original 16 transplanted mussels (T-VA-400 d), and 9 of the 16 control mussels in Lake Opasatica (C-OP-400 d). We suspect that the mortality in the C-VA-400 d group was precipitated by marking the molluscs, because we inadvertently pierced several of the rather thin shells during the engraving step. Fortunately, this marking artifact only affected the C-VA-400 d group, as C-VA specimens destined to be collected at 0, 5, 14, 30, 60, and 90 d were not marked.

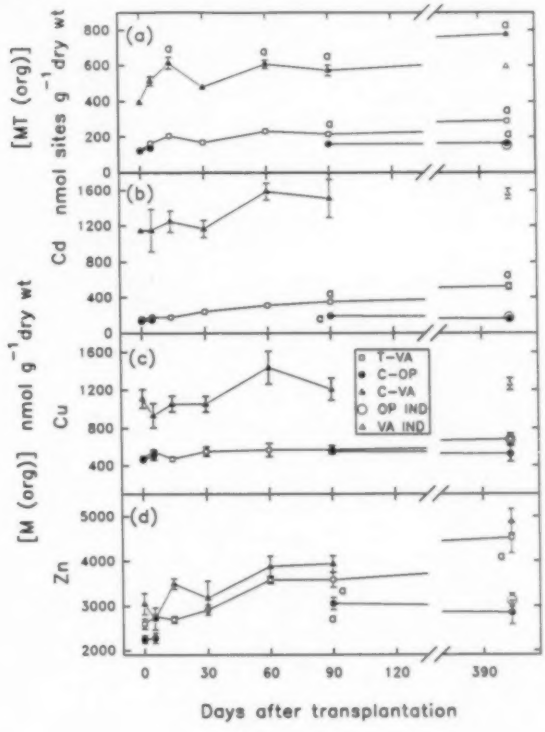
We also evaluated the enclosure and marking effect on shell growth by temporarily removing the molluscs in the C-OP-400 d and C-VA-400 d enclosures after 90 d, i.e., towards the end of the first growing season. These individual molluscs were collected by divers, measured, and returned to their enclosures. We compared the shell increments of the marked specimens from groups C-OP-400 d and C-VA-400 d with the expected shell increments for the same individuals, if they had been left unmarked outside the enclosures, for the same period of time. The expected shell increments were assessed by using the Von Bertalanffy shell growth equation of each of the bivalve populations from lakes Opasatica and Vaudray (Fig. 2*b*). We assumed that the period of active growth averages 120 d per year in the Rouyn-Noranda area, based on the average number of days between the spring and fall turnovers (F. Girard, Ministère du Loisir, de la Chasse et de la Pêche du Québec, Direction régionale de l'Abitibi-Témiscamingue, 180 boulevard Rideau, suite 180, Rouyn-Noranda, QC, J9X 1N9, personal communication). For the first 90 days of the experiment, the specimens would reach 75% of their annual growth increments. Specimens from the group C-OP-400 d grew on average 1.74 ± 0.26 mm (mean ± SE) as opposed to the expected increment of 5.30 ± 0.20 mm

Table 3. Fate of bivalves during the transplantation experiment: C-OP and C-VA correspond to specimens kept in enclosures in their source lakes, Opasatica and Vaudray, respectively; the T-VA group corresponds to the bivalves transferred from Lake Opasatica to Lake Vaudray. Initially (day 0) 16 individuals were present in each set of two enclosures.

	Missing individuals		
Day of collection	C-OP	T-VA	C-VA
0	0	0	0
5	1	0	0
14	—[a]	0	0
30	—[a]	0	0
60	—[a]	3	0
90	6[b]	5[b]	2[b]
400	6[c]	2[c]	15[c]

[a]No collections were planned for these days.
[b]On this occasion (17 September 1990), the water temperature was ≈10°C. It is possible that some individuals had already burrowed into the sediments for the winter.
[c]These enclosures were also inspected on day 90: missing specimens for the C-OP group = four, including two live specimens found close by but outside the enclosures; missing specimens for the T-VA group = zero; missing specimens for the C-VA group = nine dead (empty shells found in the enclosure).

Fig. 4. Variations of MT, Cd, Cu, and Zn concentrations over time in whole transplanted and control bivalves (note different scales). Each point is the mean ± SE of the replicate samples. Lowercase letters indicate, for a group in a given treatment, means that are significantly different from the mean at 0 d. For the transplanted mussels (T-VA), lowercase letters indicate means that are significantly different from both the C-OP replicates collected on the same dates and the T-VA replicates at 0 d ($P < 0.05$). The high MT concentration for the group C-VA-400 d is discounted because it was obtained for a single individual that exhibited anomalously high Cd and Cu concentrations in the mantle and in the remainder. Only one specimen of the C-VA-400 d group survived to the end of the experiment.



calculated for molluscs if they had been left unmanipulated in their natural environment. Likewise, specimens of the group C-VA-400 d grew on average 0.99 ± 0.46 mm as opposed to the expected increment of 4.38 ± 0.14 mm. Thus, for each of the two groups, a strong enclosure and marking effect on shell growth was detected ($P < 0.001$). Similar conclusions were reached for a subsequent manipulation carried out during summer 1992, in which specimens were marked more gently by attaching plastic labels to the posterior margin of the valves with underwater glue (Y. Couillard, unpublished data).

Hanson et al. (1988) restricted specimens of *P. grandis* to one depth zone, by the use of enclosures, in Lake Narrow, Alberta, from June 1985 to August 1986. Mussels reared at 7 or 5 m grew more slowly than mussels reared at 1 and 3 m. Differences in growth were strongly correlated with the water temperature measured for the different depths. Given these results, a possible explanation for the apparent enclosure and marking effect on shell growth observed with our experimental design would be that bivalves in their natural environment grow faster than bivalves kept in enclosures because they are able to migrate to microenvironments more favourable to growth such as a particular substrate type, warmer waters, etc. At our two sites, bivalves need to migrate less than 100 m to move from 5 m, the depth at which the enclosures were placed, to 1 m. A shortage of food in the enclosures does not seem to be a plausible explanation for the enclosure and marking effects on shell growth because, as mentioned earlier, condition indices were similar for the enclosed molluscs and for the free-living indigenous specimens (Fig. 3).

Temporal changes in metallothionein and metal concentrations during the transplantation experiment

Figure 4 shows temporal profiles for MT and metal concentrations in whole organisms for all three treatments, i.e., the transplanted bivalves plus the control bivalves at each site. The main statistical features concerning the temporal changes of [MT] and [M] for the individual body parts are summarized in Tables 4 and 5.

Table 4. Statistical analysis of metal (Cd, Zn) bioaccumulation and metallothionein (MT) induction in transferred bivalves.

Body part	[MT] (Day No.)	[Cd] (Day No.)	[Zn] (Day No.)
Gills	90*, 400**	90*, 400**	N.S.
Mantle	400**	90**, 400**	400*
Digestive gland	90*, 400**	90**, 400**	90*, 400**
Remainder	400**	90**, 400**	90*, 400**
Reconstituted organism	90*, 400**	90**, 400**	90*, 400*

Note: Collection days are indicated for which the mean metallothionein or mean tissue metal concentrations of the T-VA replicates were significantly different from (*i*) the C-OP replicates collected at the same occasions and (*ii*) the T-VA replicates at 0 d. (*, $P < 0.05$; **, $P < 0.01$). Explanation of the sample code: T-VA = specimens transferred from the less contaminated Lake Opasatica (OP) to the more contaminated Lake Vaudray (VA); C-OP and C-VA = specimens kept in enclosures in their source lakes OP and VA, respectively.

Metallothionein

At each site, seasonal variations in organism MT levels were insignificant ($P > 0.05$) relative to the marked differences between MT concentrations in the bivalves from the two sites (C-VA vs. C-OP; VA-IND vs. OP-IND). In effect, only moderate seasonal variations in [MT(org)] were observed for control mussels from lakes Vaudray and Opasatica (C-VA and C-OP; Fig. 4*a*); for Lake Vaudray, the ratio of the highest mean value obtained (14 d) to the lowest mean value (0 d) was 1.55[2] whereas for Lake Opasatica the ratio was 1.34. Differences between C-OP and C-VA mean MT concentrations at 0, 5, 90, and 400 d were always highly significant ($P < 0.001$). Similar statistical conclusions hold for the body parts.

Specimens transplanted from Lake Opasatica to Lake Vaudray (T-VA) showed a slow but steady increase in metallothionein concentrations with time. The ratio of the mean highest MT level (400 d) to the mean lowest MT level (0 d) reached 2.45 (Fig. 4*a*). Ignoring any possible enclosure effect on MT kinetics, and assuming a linear increase in MT concentrations with time all year long, we calculate that it would have taken two more years for the transplanted molluscs to reach the MT levels found in indigenous bivalves from Lake Vaudray. Because marked bivalves kept in enclosures grew more slowly than their congeners outside the enclosures, we cannot exclude the possibility that the experimental design may also have affected the dynamics of metal accumulation and perhaps MT biosynthesis.

Significant increases in [MT] in the gills, digestive gland, and in the whole organism were first noted at 90 d and persisted until the end of the experiment (Fig. 4*a*; Table 4). In contrast, increases in [MT] in the mantle and in the remainder were not detectable in the transplanted bivalves before 400 d (Table 4). Malley et al. (1993) analyzed specimens of *P. grandis* from a lake where epilimnetic mean total Cd concentrations were raised purposely from 0.01 to 0.66 nM. They observed that the gills, mantle, visceral mass, foot, and kidney all produced MT in response to the increased metal exposure. Our results are consistent with these findings.

[2] The high MT concentration observed at 400 d is discounted because it was obtained for a single animal that exhibited unusually high Cu and Cd concentrations in the mantle and in the remainder. MT and metal concentrations in this organism were excluded from the statistical analyses.

Table 5. Correlations (Pearson's *r*) between metallothionein (nmol metal binding sites·g^{-1} dry wt.) and metal concentrations (nmol·g^{-1} dry wt.) in the tissue replicates of *P. grandis* for the different treatments of the transplantation experiment.

Treatment	Metal: Cd	Cu	Zn
Gill MT			
T-VA	0.76***	−0.04	0.66***
C-OP	−0.44	−0.51	0.04
C-VA	0.27	0.21	0.31
Mantle MT			
T-VA	0.70***	0.41*	0.64***
	*0.64***	*0.36**	*0.58***
C-OP	0.59*	0.19	0.50
C-VA	0.49*	0.19	0.22
Digestive gland MT			
T-VA	0.56**	−0.24	0.57**
	*0.48***		*0.42**
C-OP	0.57*	−0.18	0.20
	0.40		
C-VA	0.15	−0.45*	0.30
Remainder MT			
T-VA	0.75***	0.12	0.78***
	*0.75***		*0.78***
C-OP	0.73**	0.36	0.72**
C-VA	0.71***	0.59**	0.70***
	*0.53***	*0.32*	*0.44**
Reconstituted organism MT			
T-VA	0.82***	0.58**	0.86***
	*0.77***		*0.82***
C-OP	0.59*	0.16	0.60*
C-VA	0.61**	0.39	0.46*
			0.37

Note: Partial correlations between [MT] and [M], holding the tissue dry weight constant, are indicated (*italic underlined*) where significant relationships between tissue dry weights and [MT] and [M] have been detected (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Cadmium

Mean whole body Cd, [Cd(org)], for control bivalves from lakes Vaudray and Opasatica (C-VA and C-OP) did not vary markedly during the transplantation experiment (Fig. 4*b*). Ratios of the mean highest [Cd(org)] to the mean lowest [Cd(org)] for the C-VA and C-OP groups were both equal to 1.4. In contrast, mean [Cd(org)] increased 3.3-fold from 0 to 400 d for the transplanted bivalves (T-VA). After 400 d, the mean [Cd(org)] in the T-VA replicate sample (525 nmol·g^{-1} dry wt.) was still only about one third of

the whole organism Cd level in the indigenous Lake Vaudray bivalves. Statistically significant increases in [Cd] for all body parts were first observed for the 90 d sample and persisted until the end of the experiment (Fig. 4*b*; Table 4).

Copper

As might have been anticipated from the similarity in the ambient dissolved [Cu] at the two sites (Table 1), [Cu] in transplanted bivalves (T-VA) did not significantly increase relative to the control bivalves in Lake Opasatica (Fig. 4*c*). However, Cu levels in the control or indigenous bivalves from Lake Vaudray (C-VA or VA-IND) were about twice those of control or indigenous specimens from Lake Opasatica (C-OP or OP-IND). The difference in whole-body concentrations was attributable to anomalously high gill [Cu] in specimens originating from Lake Vaudray (overall means for gill tissue: C-VA: ≈225 $\mu g \cdot g^{-1}$ dry wt.; C-OP: ≈80 $\mu g \cdot g^{-1}$ dry wt.); Cu levels in the other body parts were similar for C-VA and C-OP bivalves. The higher Cu concentrations in the Lake Vaudray molluscs may be related to differences in the water chemistry of the two lakes; Lake Vaudray has lower Ca concentrations, lower alkalinity, and lower pH than Lake Opasatica (Table 1).

Zinc

Zinc concentrations in the bivalves differed less than did dissolved and sediment-bound [Zn] at the two sites (Table 1). Overall means for [Zn(org)] in the transplantation experiment were 235 and 171 $\mu g \cdot g^{-1}$ dry wt., respectively, for the C-VA and C-OP bivalves. No statistical difference existed between the two groups at t = 0, 14, or 90 d ($P > 0.05$); a statistically significant difference was, however, noted between Zn concentrations in the groups OP-IND and VA-IND at 400 d (Fig. 4*d*). In marked contrast to the slow Cd dynamics described earlier, Zn concentrations in all body parts of the transplanted bivalves (T-VA) rapidly attained values similar to those in indigenous bivalves from Lake Vaudray (Table 4).

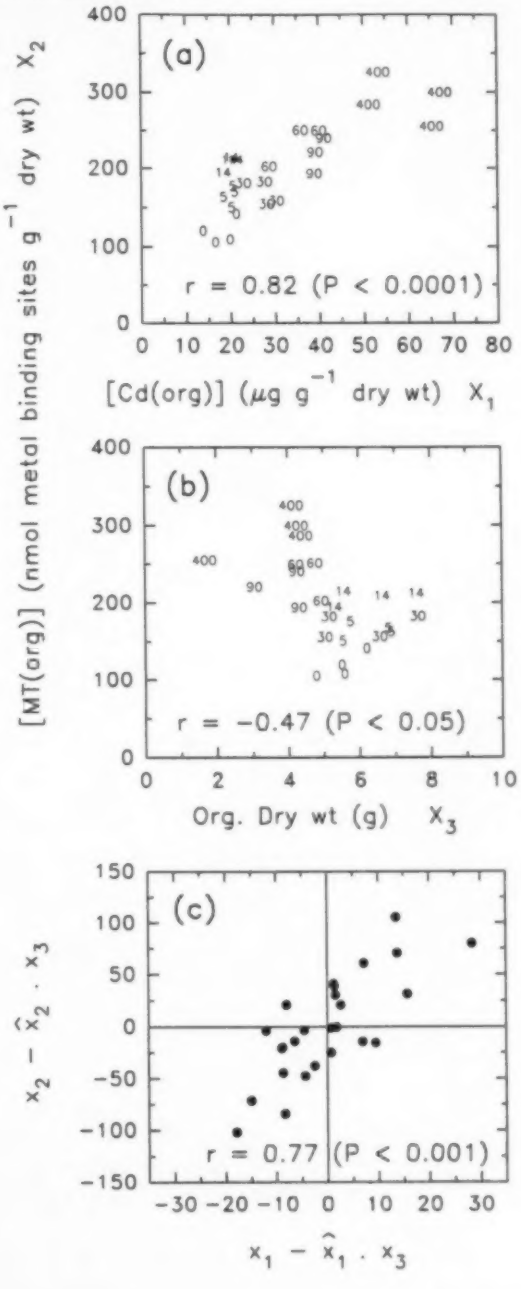
These observations are consistent with the status of Zn as an essential trace element and the existence of some form of regulation of Zn availability for Zn-dependent functions. Similar conclusions were reached by Langston and Zhou (1986), who observed that Zn concentrations in the gastropod *Littorina littorea* did not respond to fluctuations in environmental Zn contamination along the coasts of Great Britain.

Relationships between metallothionein and tissue metal concentrations

Transplanted bivalves

Relationships between MT and tissue concentrations of Cd, Cu, or Zn were examined for bivalves transplanted from Lake Opasatica to Lake Vaudray (T-VA; Fig. 5, Table 5). For the body parts considered separately, or in combination (reconstituted whole organism), [MT] were significantly correlated with [Cd] in the corresponding body parts (e.g., compare points labelled 0, 5, 30, 60, 90, and 400 in Fig. 5*a*). Whereas [MT] and [Cd(org)] increased, organism dry weights decreased during the course of the

Fig. 5. (*a*) and (*b*): Scatterplots of metallothionein concentrations (X_2) versus Cd concentrations (X_1) or organism dry weights (X_3) of transplanted bivalves. Each number corresponds to the day of sampling of the replicate samples. (*c*) Scatterplot of the partial correlation between metallothionein (X_2) and Cd concentrations (X_1) of the transplanted bivalves, holding organism dry weight constant (X_3). Solid circles are the organism individual replicates. Lines indicate lack of departure of an observation from its estimation by X_3.


(a)
r = 0.82 (P < 0.0001)
[MT(org)] (nmol metal binding sites g^{-1} dry wt) X$_2$
[Cd(org)] (μg g^{-1} dry wt) X$_1$
(b)
r = −0.47 (P < 0.05)
Org. Dry wt (g) X$_3$
(c)
r = 0.77 (P < 0.001)
x$_2$ − $\hat{x}_2$. x$_3$
x$_1$ − $\hat{x}_1$. x$_3$

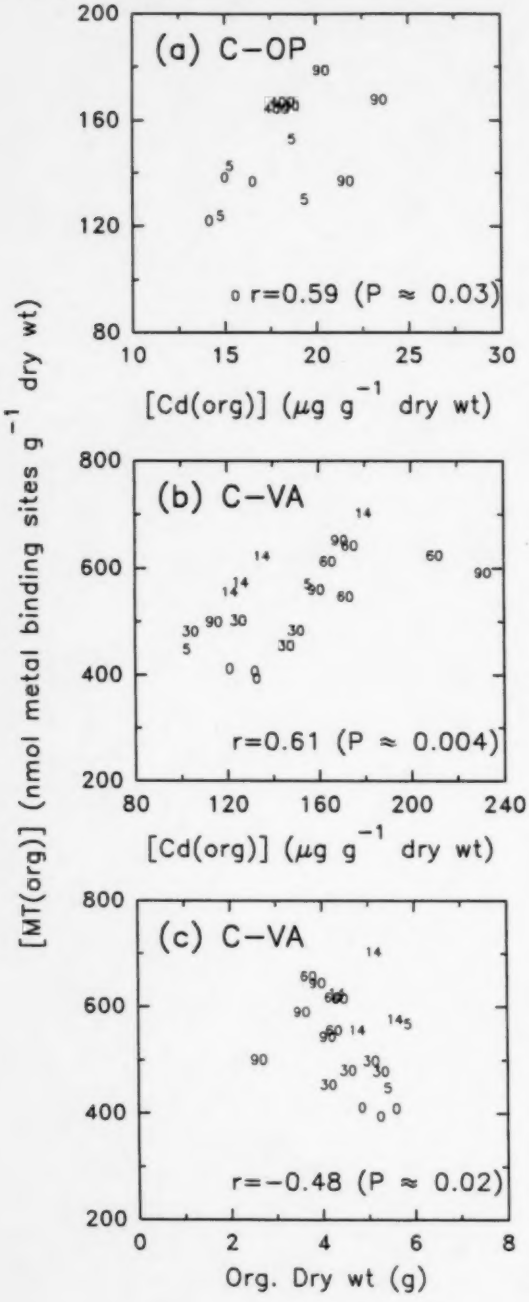

(a) C-OP
r=0.59 (P ≈ 0.03)
[Cd(org)] (μg g⁻¹ dry wt)
(b) C-VA
r=0.61 (P ≈ 0.004)
[Cd(org)] (μg g⁻¹ dry wt)
(c) C-VA
r=−0.48 (P ≈ 0.02)
Org. Dry wt (g)
[MT(org)] (nmol metal binding sites g⁻¹ dry wt)

Fig. 6. (*a*) and (*b*): Scatterplots of metallothionein versus cadmium concentrations of control bivalves from lakes Opasatica (C-OP) and Vaudray (C-VA), respectively. (*c*) Scatterplot of metallothionein concentrations versus replicate dry weights of control bivalves from Lake Vaudray. Each number indicates the day of collection of the replicate samples.

experiment (Fig. 5*b*). We initially suspected that the relationship between [MT] and [Cd] might be spurious, caused by the decreases in organism weight. However, partial correlation analysis, holding organism dry weight constant, showed that the correlation between [MT(org)] and [Cd(org)] within the T-VA group remained significant (Fig. 5*c*).

For all body parts except the gills, and for the reconstituted whole organism, we also found significant correlations between [MT] and [Zn] in the corresponding body parts, holding dry weight constant (Table 5, T-VA). This result is in apparent contrast to our earlier study of the spatial variability of MT (Couillard et al. 1993). In the earlier study we examined MT and Cd, Cu, and Zn levels in specimens of *P. grandis* from 11 lakes of the Rouyn-Noranda area (including the two lakes of the present study). MT concentrations in the gills, in the remaining tissues (whole organism minus gills and digestive gland) and in the whole organism were significantly correlated ($P < 0.01$) with tissue [Cd]. In contrast, correlations between [MT] and tissue levels of Zn were either weak or nonexistent. In addition, in a laboratory exposure experiment Roesijadi et al. (1988) found Zn to be ineffective as an inducer of these proteins in the marine mussel *Mytilus edulis* (in contrast to Cd and Cu). We hypothesize that the relation between [MT] and [Zn] in the present transplantation experiment is an artifact and suggest that if the experiment had been prolonged, organism [Cd] and [MT] would have continued to increase whereas organism [Zn] would have remained more or less constant as it had already reached levels found in indigenous bivalves from Lake Vaudray. As a result the relationships between organism [Zn] and [MT] would weaken over a longer time frame.

We thus suggest that MT concentrations in the transplanted bivalves (T-VA) increased in the course of the transplantation as a result of organism weight losses and, to a greater extent, as a result of induction caused by the bioaccumulation of Cd (Fig. 5). The response time of MT levels in transplanted *P. grandis* to a change in metal exposure was less than 90 d and increased levels of MT persisted thereafter until the end of the experiment. This dose–response relationship for Cd is also observed spatially along a contamination gradient. In an earlier study (Couillard et al. 1993) we noted a strong correlation between MT concentrations in *P. grandis* and Cd^{2+} concentrations at the sediment–water interface. No such correlations were observed with Cd, Cu, or Zn in other abiotic compartments. Thus, the results of these two studies suggest that MT satisfies criterion 3 mentioned in the Introduction, i.e., a concentration-dependent response.

Control bivalves

Within the groups of control mussels, C-OP and C-VA, MT concentrations were correlated with tissue Cd and Zn levels. However, these correlations due to seasonal variations were weaker than for transplanted mussels (T-VA), and were not systematically observed in gills, mantle, and digestive gland (Table 5). In Lake Vaudray, changes in dry weights had a significant effect on the seasonal variations of [MT], [Cd], and [Zn] in the whole organism and remainder (C-VA control bivalves; Table 5,

Fig. 6*c*). This was not the case with control bivalves from Lake Opasatica. Note that, because of the covariations with dry weight, correlations between remainder [MT] and [Cu], and between organism [MT] and [Zn], are spurious (group C-VA; Table 5).

Strong seasonal variations in metal concentrations have been reported for marine bivalves (Cain and Luoma 1990; Boyden and Phillips 1981). These variations were largely related to changes in soft tissue weight, which in turn were dependent (Boyden and Phillips 1981) or independent (Cain and Luoma 1990) of the gametogenesis–spawning cycle. Fowler et al. (1986) have shown that production of Cd-binding protein (at present defined as CdBP) in the oyster *Crassostrea virginica* was related to water temperature and to the reproductive cycle on the east coast of United States. Production of CdBP was at a minimum in winter and increased to a maximum during gonadal development and spawning from June to August. In addition, Cd associated with the protein was displaced by Cu during the late prespawning and spawning periods. Similarly, we observed that [MT(org)] of C-OP and C-VA bivalves tended to be high in specimens carrying eggs or immature glochidia in gill marsupia, i.e., 60 d (20 August 1990), 90 d (17 September 1990), and 400 d (24 July 1991) after transplantation (Fig. 6). Monitoring of indigenous mollusc populations for several consecutive years might help to determine how annual growth and gametogenesis–spawning cycles affect tissue MT and metal concentrations, as well as the metal competition for MT.

If MT is to be used as a biomarker or prior metal exposure and metal-induced stress, intrasite seasonal variations in its concentrations should be less important than intersite differences. The relatively modest seasonal variations in the MT levels of control bivalves compared with the same variations in transplanted bivalves are, therefore, reassuring. Temporal variations in [MT] in populations of *P. grandis* from other environments with more complex hydrologic and geochemical processes might, however, be more appreciable. In addition, longer continuous records of this kind are needed if we are to be able to account for the effects of endogenous and exogenous factors on MT levels in indigenous populations (criterion 4).

Concluding remarks

Most of the evidence supporting the notion that aquatic organisms synthesize MT as a defence against toxic metals has been derived from laboratory experiments. However, the metal exposure conditions shown to induce MT synthesis in these laboratory experiments are often environmentally unrealistic: short exposure times and metal concentrations orders of magnitude higher than those found in even the most contaminated aquatic systems where the studied organisms are found. The present habitat-swap experiment provides convincing field-based evidence for MT induction in a representative freshwater invertebrate, the bivalve *P. grandis*, in response to an abrupt but realistic increase in metal exposure (Cd, Zn); in our study area, Cd is the key metal to which metallothionein levels in *P. grandis* respond. The dynamics of the in situ response were, however, rather slow; after 400 d Cd and MT levels in specimens transplanted to the more contaminated site were only about one third as high as those in the indigenous molluscs present in the contaminated lake. Given the observed effect of enclosure on bivalve growth, we cannot exclude the possibility that the experimental design may also have affected the dynamics of Cd accumulation and perhaps MT biosynthesis. It is, however, reassuring that the in situ response of tissue Zn levels was rapid.

The slow increase of MT concentrations in the transplanted bivalves does not necessarily mean that MT induction/synthesis is slow. Overall changes in MT concentrations in *P. grandis* integrate both biosynthetic and degradative processes. Induction of MT synthesis might well have been rapid in the T-VA replicates, but owing to the inevitable biological variability of the controls, this phenomenon could not be demonstrated statistically before day 90. To comprehend MT biosynthesis and degradation processes fully in such an in situ experiment would require studies at the molecular level.

The present field transplantation experiment was designed to evaluate the potential of MT in *P. grandis* as a biochemical indicator of metal exposure and metal-induced stress. To evaluate this potential we can consider the data with respect to the five criteria proposed for such indicators by Haux and Förlin (1989) and by Stegeman et al. (1992) (see Introduction). The temporal correlation of MT and metal (Cd) concentrations in the transplanted bivalves suggests that MT conforms to criteria 2 and 3 for such indicators, namely that the indicator should respond in a concentration-dependent manner to changes in ambient levels of a particular contaminant or class of contaminants. Moreover, the relatively modest seasonal variations of the MT levels in the control bivalves suggest that characteristics related to the basic biology/physiology of *P. grandis*, e.g., dietary regime, growth and development, and reproduction, are less important than changes in metal bioavailability as sources of variation in [MT]. This is a particularly important requirement if MT is to satisfy criterion 4: factors, endogenous as well as exogenous, affecting the indicator should be known so that sources of uncontrolled variation can be minimized.

To complete this evaluation of MT as a biochemical indicator, we should consider links between the MT status of the organism and its general health, as well as relationships between MT responses and possible effects at higher levels of biological organization. These questions, and the compliance of MT with respect to criteria 1 and 5, are examined in Couillard et al. (1995).

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Field transplantation of a freshwater bivalve, *Pyganodon grandis*, across a metal contamination gradient. II. Metallothionein response to Cd and Zn exposure, evidence for cytotoxicity, and links to effects at higher levels of biological organization

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Abstract: To examine links between the metallothionein (MT) status of an organism and its general health, we transplanted adult specimens of the freshwater bivalve *Pyganodon grandis* (formerly *Anodonta grandis*) from a less to a more contaminated lake in the mining area of Rouyn-Noranda, in northwestern Québec. The transplanted bivalves were maintained in open enclosures placed in the bottom sediments of the contaminated lake; in addition, indigenous specimens were maintained in control enclosures in their lake of origin. Up to 16 individuals were removed from pairs of enclosures at times $t = 0$ (June 1990), 5, 14, 30, 60, 90, and 400 d. Excised gill tissue was analyzed for metallothionein, Cd, Cu, Zn, Ca, and malondialdehyde (MDA), a product of lipid peroxidation. Metal partitioning in the gill cytosol, as determined on a subset of gill samples from transplanted molluscs, changed markedly during the experiment. After 400 d, Cd was present in the low molecular weight fraction of the gill cytosol, and symptoms of cellular toxicity were detected in the transplanted molluscs (elevated [MDA] and [Ca]). At the whole organism level, the marked transplanted bivalves grew more slowly over the 400-d experiment than did marked control bivalves in Lake Opasatica, and their condition index deteriorated over time.

Résumé : Des spécimens adultes du bivalve d'eau douce *Pyganodon grandis* (anciennement *Anodonta grandis*) ont été transplantés d'un lac relativement peu contaminé vers un lac très contaminé en Cd et en Zn, dans la région minière de Rouyn-Noranda (Québec). Les bivalves transplantés et témoins (gardés dans leur lac d'origine) furent confinés dans des enclos ouverts enfoncés dans les sédiments littoraux. Jusqu'à 16 individus furent retirés des enclos aux temps 0 (juin 1990), 5, 14, 30, 60, 90 et 400 jours. Les branchies, choisies comme organe cible, furent analysées pour la métallothionéine (MT), des métaux (Cd, Cu, Zn, Ca) et la malondialdéhyde (MDA; produit de la peroxydation lipidique des membranes). La répartition subcellulaire du Cd dans le cytosol des branchies, tel que déterminée pour certains mollusques transplantés, montrait des changements importants au cours de l'expérience. Après 400 jours, l'apparition de symptômes de toxicité cellulaire dans les branchies des mollusques transplantés ([MDA] et [Ca] élevées) coïncida avec l'association du Cd avec des ligands de faible poids moléculaire dans le cytosol branchial. La croissance moyenne sur 400 jours des bivalves transplantés, marqués à cet effet,

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était significativement ralentie par rapport à celle des bivalves témoins marqués maintenus dans le lac le moins contaminé. De plus, l'indice de condition de ces mollusques transplantés se dégrada dans le temps.

Introduction

Metallothionein (MT) is a low molecular weight, cysteine-rich metal-binding protein with high affinity for groups IB and IIB metal ions. Given its molecular properties and our present knowledge of its role in metal uptake, transport, storage, and excretion, MT offers considerable potential as a contaminant-specific biochemical indicator of metal exposure and (or) stress (Roesijadi 1992; Stegeman et al. 1992). Possible approaches include

(1) *Direct measurement of [MT] as an indicator of prior exposure to toxic metals.* In this case, it is assumed that basal levels of MT are low, and that any increase in concentration in an animal above these low levels is attributable to the induction of MT in response to an influx of toxic metals. Measurements of MT would in principle distinguish between the toxicologically significant intracellular fraction of metals and metals bound in unavailable form (Roesijadi 1981).

(2) *Examination of the distribution of toxic metals in cytosolic ligand pools as a means of evaluating metal stress at the biochemical level.* It has been suggested that the excessive accumulation of metals beyond the binding capacity of available MT should result in their binding to other intracellular ligands, a phenomenon termed spillover: metals bound to these other ligands are considered to be capable of exerting cellular toxicity (Brown and Parsons 1978). In principle, this condition could be considered as symptomatic of metal stress and would be amenable to detection.

Our previous field work has shown that metallothionein concentrations in the freshwater bivalve *Pyganodon grandis* (formerly *Anodonta grandis*) respond in a concentration-dependent manner to ambient levels of Cd. This dependence was observed both spatially (interlake comparison along a contamination gradient; Couillard et al. 1993) and temporally (transplant experiment; Couillard et al. 1995). We concluded that MT is a promising biomarker of prior metal exposure (approach (1) above). Similar conclusions have been reached by other workers, based on laboratory investigations (Stegeman et al. 1992; NRCC 1985).

In contrast, field investigations of the validity of the second possible use of MT, i.e., as a biochemical indicator of metal-induced stress, are only slowly appearing in the literature (Klaverkamp et al. 1991; Brown et al. 1987; Johannson et al. 1986). Indeed, almost all the evidence for spillover in aquatic organisms has been derived from laboratory experiments (Jenkins and Mason 1988; Sanders et al. 1983) where the test organisms were exposed to (unrealistically) high metal concentrations.

In the present study, we carried out a field experiment designed to evaluate the potential of metallothionein as a biochemical indicator of metal-induced stress in *P. grandis*. Specimens were transplanted from a relatively unpolluted lake to a lake contaminated by Cd and Zn. To detect symptoms of cellular toxicity and test a hypothetical model of cytotoxicity, we monitored MT, cytosolic [Ca], and malondialdehyde (MDA; a product of lipid peroxidation) and determined the subcellular partitioning of cytosolic metals. Similarly, to investigate the relation between MT status and effects at higher levels of biological organization, we determined condition indices and shell growth rates of the specimens (organism response), as well as other characteristics of the indigenous bivalve populations involved in the transplant experiment.

The role of MT in the detoxification of excess trace metals and in the health maintenance of *P. grandis* has been evaluated within the framework of a hypothetical model of cytotoxicity (Viarengo 1989; Younes and Siegers 1984). In this model, [cytosolic metal]/[MT] ratios are taken to reflect the quantity of the metal available to express cytotoxicity (Fig. 1, step 1). The lipid peroxidation process (step 2) can be broadly defined as an oxidative deterioration of polyunsaturated lipids in the cellular membranes, initiated by reactive species of oxygen such as O^{2-}, $OH^{\cdot}$, $O_2^{\cdot}$ (Bus and Gibson 1979). Non-MT-bound nonessential metals, as well as non-MT-bound essential metals present in excess, would be expected to enhance the lipid peroxidation process, resulting in increased levels of malondialdehyde, a by-product of this peroxidation (Sunderman et al. 1985). It is well established that Cd and Cu, in particular, can stimulate this process, though by very different mechanisms (Hendry et al. 1992; Gill et al. 1990; Viarengo et al. 1990); Cu reacts with H_2O_2 directly, generating reactive oxygen species via Fenton-like reactions, whereas Cd, a redox-insensitive metal, acts by depleting the -SH antioxidant pools. In the case of sustained aggression by metals, several defence systems against oxidative damage would be impaired. A depletion of glutathione (GSH), the cell's main antioxidant, might be anticipated (step 3). Reactive oxygen species as well as intruding metals would alter the Ca-extruding systems of the plasma membrane, e.g., by interacting with -SH groups in the Ca-Mg ATPase (Viarengo 1989; e.g., with Cd: Verbost et al. 1988; with Cu: Viarengo et al. 1988). At this stage (step 4), an accumulation of Ca in the cytoplasm would occur; calcium-mediated functions of the cell would be impaired and eventually this would lead to cell death (step 5).

Several criteria have been proposed for evaluating the adequacy of various biochemical indicators (Stegeman et al. 1992; Haux and Förlin 1989). Criteria are numbered below because we refer to them by their numbers in this text and in the accompanying article.

(1) The indicator should present an early warning capacity, i.e., the biochemical response should be predictive of effects at higher levels of biological organization (population, community) and should precede them.

(2) It should be specific to a particular contaminant or a class of contaminants.

(3) It should respond in a concentration-dependent manner to changes in ambient levels of the contaminant.

(4) Endogenous and exogenous factors that affect the

indicator should be known, so that sources of uncontrolled variation can be minimized.

(5) The indicator should be related to the health or fitness status of the organism.

The present study was designed to investigate criteria 1 and 5. However, we shall use all five criteria in our final evaluation of MT as a biomarker.

Materials and methods

Study area and experimental design

The transplant experiment was carried out in two lakes in the mining area of Rouyn-Noranda in northwestern Québec. This experiment was designed to follow changes associated with increased metal exposure as a function of time. The two lakes selected differed widely in their levels of contamination (Cd, Zn), and with respect to MT concentrations found in their indigenous mollusc populations, but they shared similar ecological characteristics (Couillard et al. 1995).

Specimens of the freshwater bivalve *P. grandis* of a nominal length of 8 cm (4–6 years old) were transferred from the relatively unpolluted Lake Opasatica to the highly contaminated Lake Vaudray (treatment T-VA). Indigenous mussels were used as control groups (Opasatica: C-OP, Vaudray: C-VA). Animals were held in open enclosures placed in the bottom sediments of each lake (eight animals per enclosure). The animals transferred to Lake Vaudray (T-VA) and the Lake Vaudray controls (C-VA) were sacrificed according to the sequence 0, 5, 14, 30, 60, 90, and 400 d (t = 0: 20 June 1990). Two enclosures, that is nominally 16 animals, were sampled at each date. The C-OP control group was sampled according to the sequence 0, 5, 90, and 400 d. Bivalves destined to be collected at 400 d were marked to follow their growth. In addition, 12 indigenous molluscs were collected outside the enclosures at each site at 400 days and are identified as OP-IND (Lake Opasatica) and VA-IND (Lake Vaudray).

In coding the various groups, we have used the following conventions: C, control; T, transferred; OP, Lake Opasatica; VA, Lake Vaudray; IND, indigenous, free living. The number of days of exposure completes the code, e.g., T-VA-90 d designates molluscs transferred to Lake Vaudray and collected after 90 days exposure. The location of the lakes, the rationale for site selection and the details of the experimental plan are summarized in the companion paper in this issue (Couillard et al. 1995).

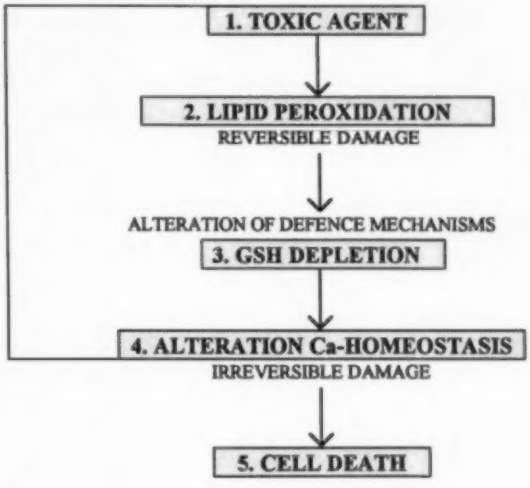
Bivalve analyses

The bivalves from all groups were dissected within 4 h of collection into four tissue groups: gills, mantle, digestive gland, and remainder. To obtain enough material for all the analyses, tissues from four bivalves were pooled yielding four replicate samples per treatment and per date, when no mortality had occurred. We computed, for each replicate, the following condition index (CI; Lucas and Beninger 1985):

$$[1] \quad CI = \frac{\text{Total flesh dry weight (g)}}{\text{Total shell weight (g)}}$$

Four pairs of demibranch gills from each replicate were retained at the time of dissection for determination of cytosolic Cd, Cu, Zn, and Ca concentrations ($[M]_{cyt}$), where

Fig. 1. Model of trace metal cytotoxicity showing the sequence of events leading to cell death after lipid peroxidation. Variables used to evaluate the model are the ratios of cytosolic metal to MT concentrations, step 1; malondialdehyde concentrations, [MDA], step 2; and cytosol [Ca], step 4. The target organs are the gills. See text for explanations. Adapted from Viarengo (1989) and Younes and Siegers (1984).



M = metal. These samples were also used for determinations of malondialdehyde concentrations [MDA] as well as for the determination of the partioning of Cd, Cu, and Zn among cytoplasmic ligands. The tissues were preserved as described in Couillard et al. (1995), until the homogenization step. One-half gram of partially thawed tissue from each replicate was homogenized with a glass homogenizer and pestle in 5 mL of ice-cold 154 mM KCl solution; this extract was analyzed the same day for MDA. The remaining tissues were homogenized in three or nine weights of ice-cold 25 mM Tris solution, adjusted to pH 7.2, with a glass homogenizer and motor-driven Teflon pestle at 80 rpm; the homogenates were immediately frozen and stored at −80°C for eventual analysis of cytosolic metals. Dissection and preservation procedures and tissue metal determinations are fully described in Couillard et al. (1995). The occurrence of MT in *P. grandis* and the MT assay used here are discussed in Couillard et al. (1993).

For each sample of a given treatment obtained at 60 d (20 August 1990) and 90 d (17 September 1990), gravid females, identified by visual inspection of the gills, were counted. Larvae extracted from the marsupium of each female were weighed. Larval replicates, obtained by pooling four specimens, were analyzed later for MT and Cd, Cu, Zn, and Ca concentrations, and their dry weight (wt.)/wet wt. ratios were determined.

Malondialdehyde determinations in gills

MDA concentrations in the gills were measured on whole tissue homogenates by the colorimetric method of Sunderman et al. (1985), which is based on the reaction of thiobarbituric acid with MDA and related chromogens. Two

Table 1. Cadmium and Cu concentrations in HPLC gel permeation fractions of gill cytosols from specimens transplanted to Lake Vaudray (T-VA) and collected after 14, 90, or 400 d.

Molecular weight fraction	Elution volume (mL)	[M] in fractions for each treatment (μg/g dry wt.) T-VA-14 d [Cd]	T-VA-90 d [Cd]	T-VA-90 d [Cu]	T-VA-400 d [Cd]
High	4–6	0.74	0	1.32	0
	6–8	0.42	0	5.62	0
	8–10	0.59	0	1.41	1.09
MT[a]	10–12	0	0	0	0
	12–14	0	6.62	1.49	0.96
Low	14–16	0	0	0	5.73
	16–18	0	0	0	0
	18–20	0	0	0	0
	20–22	0	0	0	0
Σ fractions (μg/g dry wt.)		1.74 versus 2.28	6.62 versus 4.26	9.83 versus 10.87	7.79 versus 6.33
Recovery of total $[M]_{CYT}$[b] (%)		77	155	91	123

[a]10 kDa corresponds to an elution volume of 12.6 mL.
[b]The total metal concentrations in gill cytosols were determined by plasma atomic emission spectrometry in digested subsamples of 170 000 × *g* gill homogenates.

determinations of [MDA] were conducted on each homogenate and each determination was corrected for background absorption due to reagent blanks (about 0.01 mmol MDA equivalents/g wet wt.). Mean coefficients of variation (CV) for such determinations were ≤6%.

Metal concentrations in gill cytosols

Homogenized gill tissues were thawed and centrifuged at 170 000 × *g* for 1 h at 2°C. Subsamples (2 mL) from the supernatant were acidified with 0.5% ultrapure HNO_3 (Aristar; vol/vol). Concentrations of Cd, Cu, Zn, and Ca were measured by plasma atomic emission spectrometry.

Determination of metal partioning in gill cytosol preparations

For the transplanted molluscs collected at t = 14, 90, and 400 d, a composite sample representing one quarter of the gill tissue from four mussels was analyzed. A 100-μL subsample of the 170 000 × *g* gill cytosol supernatant obtained above (nonacidified) was fractionated by high performance liquid chromatography (HPLC: Waters 600 chromatograph) on a steric exclusion column (TSK gel SW 2000, 30 × 0.75 cm) and precolumn (Biorad, 7.5 × 0.75 cm). The column was eluted with a pH 7 solution containing 10 mM Tris-HCl, 100 mM NaCl, and 0.03% NaN_3 at a flow rate of 0.5 mL/min (Micallef et al. 1992). The eluant was degassed with helium before use and periodically degassed during HPLC operation.

The following molecular weight standards (Sigma Chemical Co., St. Louis, Mo.) were used to calibrate the column: dextran (2000 kDa, V_0 = 5.7–5.8 mL), bovine albumin (67 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa), ribonuclease-*a* (13.7 kDa), cytochrome-*c* (12.4 kDa), aprotinin (6.5 kDa), vitamin B12 (1355 Da), and tryptophan (204 Da). Two-millilitre fractions were collected automatically (LKB 2211 SuperRac) up to 22 mL and analyzed for Cd and Cu by flameless atomic absorption spectrophotometry (AAS; Varian Techtron, model Spectra AA-30 equipped with a GTA-96 graphite tube atomizer). As an analytical control, a certified reference water sample (NIST, Trace elements in water, SRM No. 1643b) was diluted 1:4 in the eluant solution and analyzed by flameless AAS. After correction for the dilution factor, concentrations measured were within ±9% of the certified values for Cd and Cu. Metal concentrations in the chromatographic fractions were corrected for background metal concentrations in the eluant solution. Zn measurements were compromised by a high background in the eluant solution and, thus, are not reported. The chromatographic fractions were divided into three metal–ligand pools based on data from the molecular weight standards: high molecular weight pool (HMW), from the molecular weight cut-off of the column to 15 kDa; metallothionein pool (MT), from 15 to 3 kDa; and a low molecular weight pool (LMW), <3 kDa.

The subcellular distributions of metals in gills presented in this paper are operationally defined. Homogenization and fractionation procedures will normally cause organelle disruption and may lead to metal redistribution within cellular compartments. Gills homogenized as in our study, i.e., between a Teflon surface and a glass surface, should result in low organelle breakage (Julshamn and Andersen 1981). Competition experiments performed with commercial metallothionein (prelabelled with ^{109}Cd) and fresh bivalve cytosol extract demonstrated that no appreciable Cd exchange occurred during the 20 min preequilibration step or the subsequent chromatographic separation (Micallef et al. 1992). Thus, Cd–MT complexes in gill cytosols obtained after HPLC separation in this study can be

Table 2. Results of one-way analyses of variance applied to the temporal profiles of condition indices, [MDA(gill)], [Ca(gill cytosol)], and the [Cd(gill cytosol)]/[MT] ratio for each treatment series: T-VA, C-OP, and C-VA. Scheffe contrasts between group means at 0 and 400 d are indicated.

Variable	Treatment series	$F(df_1, df_2)$	P	Scheffe contrasts between means at 400 and 0 d
Condition index	T-VA	$F_{5,16} = 7.18$	≈0.001	Means different, $P < 0.05$
	C-OP	$F_{3,10} = 5.06$	>0.05	NS $P > 0.05$
	C-VA	$F_{4,15} = 1.33$	>0.05	
[MDA(gill)]	T-VA	$F_{6,19} = 2.52$	>0.05	
	C-OP	$F_{3,10} = 2.60$	>0.05	
	C-VA	$F_{5,18} = 0.28$	>0.05	
[Ca(gill cytosol)]	T-VA	$F_{6,19} = 13.57$	<<0.001	Means different, $P < 0.01$
	C-OP	$F_{3,10} = 3.14$	>0.05	
	C-VA	$F_{5,18} = 6.18$	<0.01	
[Cd(gill cytosol)]/[MT]	T-VA	$F_{6,23} = 2.79$	<0.05	Means different, $P = 0.05$
	C-OP	$F_{3,14} = 5.17$	<0.05	NS $P > 0.05$
	C-VA	$F_{5,18} = 8.52$	<0.001	

considered to represent the original distribution in the cytosol preparation.

Statistical analyses

Within each treatment series (T-VA, C-OP, C-VA), one-way analyses of variance were applied to test for significant differences over time of condition indices, [MDA], cytosolic [Ca] and [Cd]/[MT]. Similar analyses were used to compare measurements made in larvae from the T-VA, C-OP, and C-VA treatment groups at 90 d. Differences among group means were examined using Scheffe contrasts. The assumptions required for use of these parametric methods were generally met with the raw (nontransformed) data.

Results

Metal partitioning in gill cytosols of transplanted bivalves

The subcellular distribution of Cd in the gill cytosol of *P. grandis* was determined on three occasions during the course of the transplant experiment. Chromatographic profiles of Cd in gill cytosols of transplanted bivalves, T-VA, were obtained for a single composite sample at 14, 90, and 400 d (Table 1). For Cu, subcellular partitioning was determined only on day 90. Cadmium concentrations in several of the HPLC fractions (0.2–0.3 $\mu g \cdot L^{-1}$) were close to the analytical detection limit of 0.08 μg Cd·L^{-1}. The resulting analytical imprecision undoubtedly contributed to the variable recoveries of Cd after HPLC fractionation (77–155% of total $[Cd]_{cyt}$, as judged from mass balance calculations). Despite this variability, the HPLC profiles show important qualitative differences over the time course of the experiment.

Fourteen days after the initiation of the experiment, Cd was associated exclusively with HMW ligands in gill cytosols of transplanted bivalves. After 90 days, all the Cd had apparently shifted to fractions of moderate molecular size (15–3 kDa; Table 1) corresponding to the expected MT fraction. These results are consistent with the estimated response time of gill MT to the change in ambient Cd after transplantation (90 d; Table 4 in Couillard et al. 1995). In contrast to Cd, and in accordance with its status as an essential element, cytosolic Cu at 90 d was mainly bound (85%) to the HMW pool, which contains metalloproteins (Table 1). After 400 d, Cd appeared for the first time in fractions of low molecular weight (74% of cytosolic Cd; Table 1). The possible toxicological significance of this latter redistribution is considered in the Discussion.

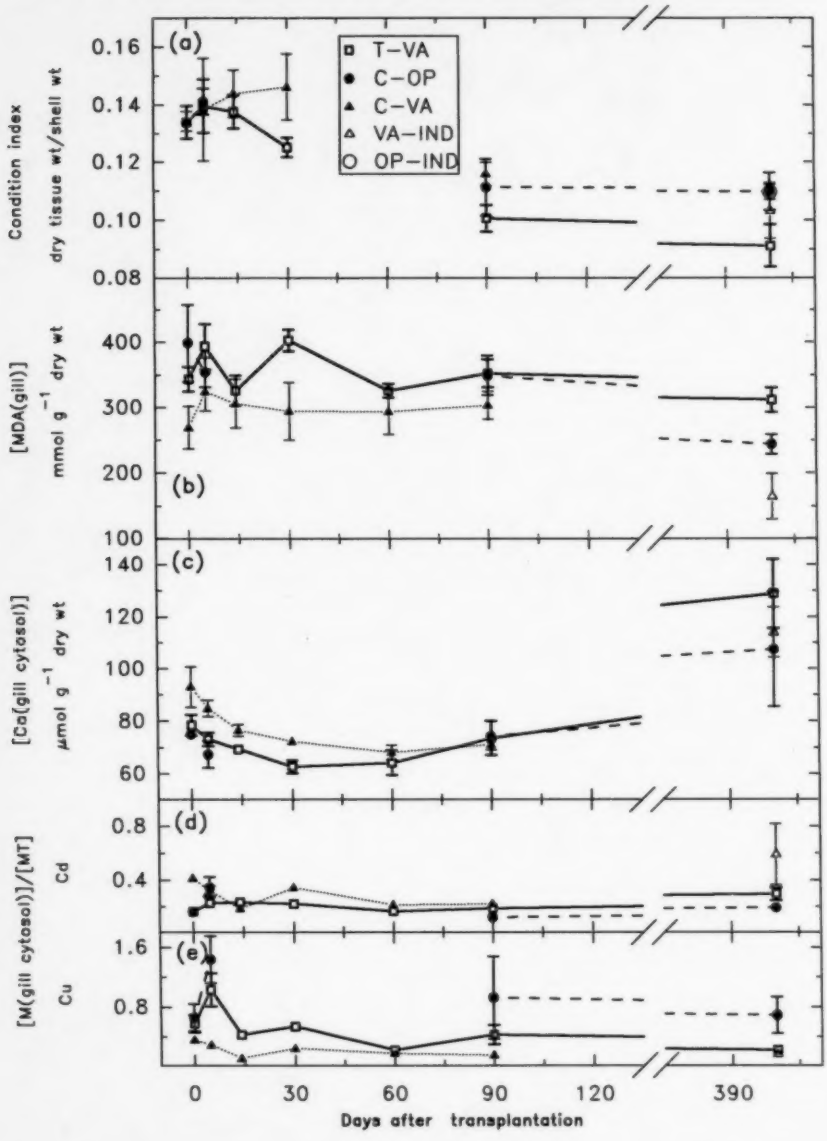
Trace metal cytotoxicity

To test the hypothetical model of trace metal cytotoxicity (Fig. 1), gills were chosen as a likely organ in which the postulated chain of cellular events might occur. Their exposure to ambient conditions is more direct than for internal organs; for example, gills accounted for 38–47% of Cd body burdens in control bivalves for this experiment, but contributed only about 21% to the organisms' total dry weight. Their contribution to MT burdens was even lower (only 11–14%). Moreover, gills of individual specimens from the indigenous population of Lake Vaudray were often deformed, whereas gills from Lake Opasatica specimens appeared more regular in shape.

Metal available to express cellular toxicity was represented by the ratio of metal concentration in the cytosol to MT concentration (see Discussion); we assume, for MT, that 1 mole of metal-binding sites as measured by the ^{203}Hg-saturation assay can bind 1 mole Cd, or 1.71 (12/7) moles Cu (Nielson et al. 1985). The implicit assumption is that higher $[M]_{cyt}$/[MT] ratios correspond to less effective metal detoxification. Lipid peroxidation was evaluated on the basis of the MDA concentration. Increases in $[Ca]_{cyt}$ were taken to indicate an increase in membrane permeability. To link these cellular events to metal toxicity at the organism level, we monitored mollusc condition indices (Eq. 1).

Temporal profiles of each of the above variables in the gills of control and transplanted bivalves appear in Fig. 2.

Fig. 2. Variations over time of condition indices, [MDA], [Ca], and [metal]/[MT] ratios in gill cytosols of transplanted and control bivalves. Each point is the mean ± SE of the replicate sample. T-VA, mussels transplanted from Lake Opasatica (OP) to Lake Vaudray (VA); C-OP and C-VA, specimens kept in enclosures in their source lakes, OP and VA respectively; OP-IND and VA-IND, specimens collected outside the enclosures in lakes OP and VA, respectively, at the end of the experiment (400 days). See text for discussion.



For the three treatments, mean [MDA(gill)] did not vary significantly with time (Fig. 2*b*; Table 2). Mean [Cu] in the gill cytosols of transplanted bivalves (T-VA) did not change significantly with time (results not shown). In addition, mean $[Cu]_{cyt}$/[MT] ratios for T-VA replicates were always <1, suggesting that transplanted specimens handled this essential metal efficiently for the duration of the transplant experiment (Fig. 2*e*).

Mean [Ca(gill cytosol)] and condition indices varied much more in the transplanted bivalves than in the controls (Figs. 2*a* and 2*c*, Table 2). Mean $[Cd]_{cyt}$/[MT] varied significantly with time in all three treatments (Fig. 2*d*, Table 2). Mean Cd concentrations in gill cytosols of transplanted bivalves were significantly higher at 90 and 400 d than the mean (±SE) value obtained for the T-VA replicate sample at 0 d ($[Cd]_{cyt}$(µg/g dry wt.) T-VA-0 d, 1.44 ± 0.19;

Table 3. Characteristics of the bivalve populations (*Pyganodon grandis*) from Lake Opasatica and Lake Vaudray, and from a reference lake. (Based on the approach of Munkittrick and Dixon (1989). See text for discussion.)

Population characteristics	Lakes		
	Vaudray	Opasatica	Reference lake
Mean age (yr)	5.23	4.88	—
Growth rate	Similar[a]		—
Condition index	0.103±0.009	0.110±0.003	0.114[b]
Fecundity (% gravid female/sample)	29	50	38[c]
Mean dry wt. larvae per bivalve (g)	0.14	0.25	—

[a]See Fig. 3*a* in Couillard et al. (1995).

[b]Reference population of *P. grandis* living in a pristine Precambrian Shield lake in northwestern Ontario. Condition index calculated from Huebner et al. (1990).

[c]Reference population of *Elliptio complanata* living in a relatively unpolluted lake in southern Québec (Downing et al. 1989).The number of gravid females per sample was computed from the percentage of females and hermaphrodites, and the relationship between the fraction of animals bearing eggs or glochidia and the allocation of male or female tissue in the gonad. Only the sample of bivalves in the size class 50–90 mm was considered for this calculation.

T-VA-90 d, 3.36 ± 0.51; T-VA-400 d, 7.06 ± 1.36; Scheffe contrasts, $P < 0.05$). For the final sampling date, the mean $[Cd]_{cyt}/[MT]$ ratio and gill cytosol [Ca] for the transplanted molluscs (T-VA-400 d) were significantly higher than for the transplanted group at 0 d. The mean condition index of the group T-VA-400 d was significantly lower than that for the group T-VA-0 d. No such significant temporal differences for these three variables were detected between control bivalves in Lake Opasatica collected at 400 and 0 d (Table 2).

Mean gill MDA concentrations in transplanted specimens at 400 d differed significantly from those in control C-OP specimens (*t*-test for small samples; $t(5) = 2.67$, $P < 0.05$). At 400 d, mean [MDA] and $[Ca]_{cyt}$ of the T-VA-400 d group were the highest of the three groups, and the mean condition index of the T-VA-400 d specimens was the lowest (Fig. 2). These observations for the final sampling date are consistent with the model of trace metal cytotoxicity proposed earlier, though the sequential nature of the model is not revealed by our results (see Discussion).

Shell growth

Changes in shell growth, as well as in condition index, were assumed to be responses at the organism level to a change in metal exposure. Mean growth of marked transplanted bivalves over the 400-d experiment was significantly lower than mean growth of marked control bivalves from Lake Opasatica ([(mean length at 400 d − mean length at 0 d)/mean length at 0 d] × 100; T-VA, 1.70%, $N = 14$, mean shell increment = 1.7 mm; C-OP, 3.9%, $N = 9$, mean shell increment = 3.9 mm; two-sample *t*-test, $P < 0.001$). For each of the two groups, this effect of transplantation is superimposed on a general enclosure and marking effect on shell growth. As discussed in Couillard et al. (1995), we attribute this general effect to the fact that marked bivalves kept in enclosures were unable to migrate to microenvironments more favourable to growth. Shortage of food in the enclosures does not seem to be a plausible explanation for the enclosure and marking effect on shell growth because condition indices were similar for enclosed and indigenous species. As both the C-OP and T-VA molluscs originated from Lake Opasatica, and both treatment groups (C-OP-400 d and C-VA-400 d) were marked and confined, we can assume that the enclosure and marking effect was similar in both samples; thus, we conclude that the decrease in growth noted for the transplanted specimens is real and is correlated with the metal contamination in Lake Vaudray.

Responses at the population level

To evaluate the effects of metal stress on the bivalve populations involved in the transplant experiment, we have adapted the approach of Munkittrick and Dixon (1989) for in situ assessment of toxicant impacts on fish populations. The principle of their approach is that a population of organisms found to be growing, reproducing, and surviving within the limits observed for a comparable reference population will be considered free from detrimental contaminant-exposure effects. Characteristics of mussel populations from our study lakes are presented in Table 3. No detectable differences in mean age or mean growth rate were noted between the Lake Opasatica and Lake Vaudray populations. Moreover, mean condition indices of these populations were similar to that of the bivalve population in our reference lake, a pristine Precambrian Shield lake in northwestern Ontario (Huebner et al. 1990). In this latter study, measured dissolved [Cd] averaged 0.01 ± 0.001 nM and gill MT concentrations in indigenous *P. grandis* were lower than in Lake Opasatica (Couillard et al. 1995). These observations suggest that adults from our transplant lakes were not experiencing metal-related toxicity.

Fecundity data in Table 3 have been obtained from the control specimens C-OP and C-VA collected at 90 d. A population of the unionid *Elliptio complanata* in a relatively

Table 4. Metallothionein and metal concentrations, $[Cd]_{T(tissue)}$/[MT] ratios and total dry weight of larvae from the T-VA, C-OP, and C-VA treatment groups at 60 and 90 days (mean ± SE). For the statistical comparisons, the groups collected at 60 days are treated separately from the groups collected at 90 days.

Variables	Treatments at 60 days†		Treatments at 90 days†		
	T-VA	C-VA	T-VA	C-OP	C-VA
MT (nmol M sites/g dry wt.)	61±5.7*a**	102±9.8*b**	26±8.5*a*	23±3.8*a*	26±2.6*a*
Cd (nmol/g dry wt.)	11±4*a*	22±4*a*	11±5*a*	5±3*a*	65±8*b***
Cu (nmol/g dry wt.)	260±20*a*	255±60*a*	620±145*a*	550±170*a*	1380±820*a*
Zn (nmol/g dry wt.)	520±30*a*	535±70*a*	470±8*a*	470±20*a*	660±30*b***
Ca (μmol/g dry wt.)	1800±140*a*	2160±130*a*	5400±430*a*	6070±200*a*	5200±14*a*
$[Cd]_{T(tissue)}$/[MT]	0.20±0.08*a*	0.22±0.06*a*	0.39±0.16*a*	0.21±0.13*a*	2.53±0.56*b***
Larval dry wt. per bivalve (g)	0.06±0.02*a*	0.04±0.01*a*	0.14±0.03*a*	0.25±0.05*a*	0.14±0.02*a*

Note: For each variable × time combination, significant differences among means are indicated by different lowercase letters, *a* and *b*. (Scheffe contrast or two-sample *t*-test: *, $P < 0.05$; **, $P < 0.01$).

†Replicate sample sizes: T-VA-60 d, 4; C-VA-60 d, 2; T-VA-90 d, 3; C-OP-90 d, 3; C-VA-90 d: 2.

uncontaminated lake in souththern Québec has been taken as our reference population (Downing et al. 1989). This bivalve has a reproduction strategy similar to *P. grandis* (ovoviviparity, Mackie 1984; common fish hosts, Clarke 1981) and the two species occur together in several lakes of the Rouyn-Noranda area (unpublished observations). Relative to Lake Opasatica and the reference lake, mussels from Lake Vaudray exhibited a lower incidence of gravid females per sample and a lower mean larval dry weight per individual, suggesting possible toxic effects on larval stages.

Further evidence for the existence of adverse effects of trace metals on the reproduction of *P. grandis* in Lake Vaudray is provided by an examination of MT and metal concentrations in larval stages. Larvae were removed from the parent demibranchs at 60 d (August 19; possibly embryonic stages) and 90 d. The high mean Ca concentrations of replicate samples at 90 d (Table 4) suggest that the larval shells were developing at that time (see Mackie 1984). At 90 d, for similar levels of MT, C-VA larvae had a ≈12-fold higher mean Cd concentration ($P < 0.01$) than C-OP larvae. C-VA-90 d larvae also showed a higher ratio, statistically highly significant, of tissue Cd to MT ($[Cd]_{T(tissue)}$/[MT]; Table 4). The situation was markedly different at 60 d, when C-VA larvae displayed, respectively, an approximately fourfold higher concentration of MT but approximately a threefold lower concentration of Cd in their tissues than at 90 d. This temporal trend was similar for larvae in the transplanted bivalves (T-VA). At 90 d, reproduction of the transplanted bivalves did not yet seem to be severely affected: the T-VA-90 d group exhibited an incidence of gravid females (5 gravid females of 11 specimens) similar to the C-OP-90 d group. For roughly equivalent MT levels at 90 d, larvae of the transplanted mussels had a higher mean Cd concentration and a lower larval dry weight per individual (not statistically different) than did larvae from control mussels in Lake Opasatica. With the exception of Zn levels in groups collected at 90 d, levels of Cu and Zn were similar for all larval groups on a given sampling date.

In principle, larval shells as well as soft tissues contributed to the Cd content of larvae samples obtained at 90 d. These shells probably did not yet possess a periostracum layer (Pennak 1989), known to have an enormous capacity to bioconcentrate trace metals in adults (e.g., bioconcentration factor for Cd in the zebra mussel (*Dreissena polymorpha*) periostracum ≈ 70 000; Bias and Karbe 1985). Lingard et al. (1992) measured Cd concentrations in isolated nacre layers of shells (periostracum removed), and in soft tissues of adult specimens of the freshwater bivalve *Elliptio complanata* from a softwater lake in Ontario. They obtained mean values of 3.3 nmol $Cd \cdot g^{-1}$, 0.5 nmol $Cd \cdot g^{-1}$, and 169 nmol $Cd \cdot g^{-1}$ dry wt. for the "nacre protein fraction," the "nacre crystal fraction," and the soft tissues, respectively.

Discussion

Metal partitioning in gill cytosols of transplanted bivalves

Roesijadi and Klerks (1989) exposed oysters, *Crassostrea virginica*, to Cd concentrations such that $[Cd^{2+}]$ fluctuated in the range of 35–40 nM for 24 d. At various times during exposure, they excised gills, exposed them to ^{109}Cd for 45 min, and studied the kinetics of Cd binding to MT and other intracellular ligands. They concluded that Cd binding in the gill is controlled by competition among available ligands displaying varying affinities for the metal, MT having the highest. Before induction of MT synthesis, estimated to have occurred at 4 d, Cd was bound predominantly to the particulate fraction and the basal MT pool, the size of which was not specified. After induction, MT became the major Cd-binding ligand and Cd initially bound to other fractions (particulate, HMW, or LMW) shifted onto MT.

The ambient Cd^{2+} concentrations and the exposure duration in the present field experiment are very different from the laboratory conditions of Roesijadi and Klerks (1989). Despite these differences, the behaviour of Cd in the gills of the transplanted bivalves, before and after induction of MT (Table 1), resembles the competitive binding of Cd described by these researchers and others (Bebianno et al. 1992; Brown et al. 1987; Hamilton et al. 1987).

Model of trace metal cytotoxicity

To detect symptoms of cellular toxicity and test a hypothetical model of cytotoxicity (Fig. 1), we monitored MT, cytosolic [Ca], and MDA and determined the subcellular

partitioning of cytosolic metals. Implicit in our testing of this model of metal cytotoxicity is the use of $[M]_{cyt}/[MT]_{cyt}$ ratios as biochemical indicators of intracellular metal availability. This hypothesis merits close scrutiny.

In the early literature on the role of MT in metal detoxification, excess cytosolic metal not bound to MT was deemed deleterious (Brown and Parsons 1978). Thus, a $[M_{cyt}]/[MT]$ ratio greater than 1 was associated with the occurrence of cytotoxicity (Hogstrand 1991). The laboratory studies at the origin of these ideas involved the exposure of organisms to single metals (e.g., Hg; Brown and Parsons 1978). However, in nature, animals are exposed to metal mixtures; hence, the $[M_{cyt}]/[MT]$ ratios expressed for an individual metal are no longer easily interpreted. In vitro studies suggest that the affinity of MT for different metals decreases in the order Hg(II) > Cu(I) > Cd(II) > Zn(II) (Roesijadi 1992). If this same sequence were applicable in vivo, then one might anticipate that Cd would be bound to MT only if the concentration of the latter exceeded the cytosolic Cu concentration (i.e., that Cu would outcompete Cd for binding sites on MT). This does not seem to be the case. For example, Brown et al. (1987) examined the cytosolic distributions of Cd, Cu, and Zn in livers of three fish species collected from highly contaminated and less contaminated southern California coastal sites. They observed for the three species at both stations that most of the cytosolic Cd (75–95%) was in the MT pool, even though significant proportions of Cu (7–50%) were bound to HMW cytosolic ligands other than MT. Similarly, in the present experiment, 90 d after the transfer of the molluscs to the contaminated lake 100% of the cytosolic Cd, but only 15% of cytosolic Cu, was associated with chromatograhic fractions including MT (Table 1; results discussed below).

Given these metal partitioning results, the ratios [Cd]/[MT], [Cu]/[MT], and [Cd + Cu]/[MT] might be of toxicological significance in relation to the physiology of the cell. We have tested the relevance of these ratios as indicators of metal availability in the cytosol in the model of trace metal cytotoxicity presented above. Ratios of [Zn]/[MT] were ignored in the statistical data treatment, because Zn on MT would presumably be easily displaced by Cu or Cd. Given its status as an essential element, the association of Zn with enzymes or with cytosolic biomolecules other than MT cannot be taken as an indication of Zn toxicity.

Links between cytosolic metal distribution and cytotoxicity

To test the model of trace metal cytotoxicity, we examined temporal profiles of key variables of the model in bivalves transplanted to a contaminated lake and in indigenous bivalves put in enclosures in the source and destination lakes (Fig. 2, Table 2).

Transplanted bivalves display the highest [MDA] and $[Ca]_{cyt}$ and the lowest condition index of the three treatments at 400 d (Figs. 2*a*, 2*b*, and 2*c*: group T-VA-400 d). It is tempting to relate these apparent symptoms of cellular toxicity to the detection of Cd in the low molecular weight cytosolic fraction (based on a single composite sample; Table 1). Similar shifts in cytosolic metal distribution have been reported in the literature. For example, in specimens of the marine bivalve *Macoma balthica* collected at monthly intervals in San Francisco Bay, Johannson et al. (1986) observed that [Cu], [Ag], and [Zn] in the LMW fractions increased steadily when the MT–metal pool appeared saturated at high cytosolic metal concentrations. However, they were not able to link such cytosolic metal profiles to indications of in situ metal stress. From laboratory experiments with the polychaete *Neanthes arenaceodentata*, Jenkins and Mason (1988) observed perturbations in reproduction at 10 nM Cd^{2+}; this coincided with increased accumulation of Cd in the very low molecular weight pool in the animal cytosol.

These studies, as well as the present investigation, suggest that shifts in cytosolic metal distribution do occur and that they may eventually prove to be useful in the diagnosis of metal-induced stress. However, the toxicological significance of metal (Cd) binding to ligands in the LMW cytosolic pool must first be elucidated.

Condition of indigenous molluscs

In contrast to the negative effects of the Lake Vaudray environment on the molluscs transplanted from Lake Opasatica (growth and condition index decrease), the indigenous adult bivalves in Lake Vaudray appear to be in reasonable health despite their higher body burdens of Cd, Zn, and Cu (cf. Table 1 in Couillard et al. 1995). In effect, bivalves from Lake Vaudray seem to have been able to counter the potential toxic effects of these high gill metal concentrations (*i*) by excluding the metal from the cytosol, e.g., Cu but not Cd, and storing it elsewhere in the gill tissue and (*ii*) by increasing the levels of MT in the gill cytosol.

Indigenous bivalves	Lake Vaudray		Lake Opasatica	
Gill tissue	Cd	Cu	Cd	Cu
Tissue metal ($nmol \cdot g^{-1}$ dry wt.)	2560	3060	280	1435
Cytosolic metal ($nmol \cdot g^{-1}$ dry wt.)	245	116	19	113
Tissue MT (nmol sites$\cdot g^{-1}$ dry wt.)	410		110	

Lake Vaudray free-living (VA-IND) as well as enclosed mussels (C-VA) exhibit lower levels of gill MDA than do bivalves from the other treatments for the duration of the experiment (Fig. 2*b*; cf. Pellerin-Massicotte 1994). A similar trend was reported by Rodriguez-Ariza et al. (1992), who collected specimens of the marine bivalves *Chamalea gallina* and *Crassostrea gigas* along the South Atlantic Spanish coast. Animals from the more contaminated areas (Cd, Cu, Hg, and Pb) had higher levels of antioxidant enzymes and lower levels of MDA. Presumably, the more contaminated organisms were better protected from oxidative stress. Similar tendencies were observed for fish populations living in environments contaminated by toxic metals (antioxidant compounds: Klaverkamp et al. 1991; Brown et al. 1987).

Critique of the observed results

The present experiment does not constitute an unambiguous field validation of the hypothetical model of trace metal

cytotoxicity presented in Fig. 1. Several inconsistencies are enumerated below.

(1) Results from the HPLC fractionation suggest that transplanted bivalves face a spillover of Cd into low molecular weight ligands in the gill cytosol at the end of the experiment (group T-VA-400 d). This spillover occurs even though the $[Cd + Cu]_{cyt}$/[MT] ratio is less than 1 on day 400 in the T-VA group. Ratios <1 indicate that there should be enough MT to complex all the Cd in the gill cytosol. Moreover, the mean $[Cd]_{cyt}$/[MT] ratio for the VA-IND group is greater than for the T-VA-400 d group, but the former group does not exhibit any obvious symptoms of Cd-induced toxicity: [MDA(gill)] and $[Ca]_{cyt}$ are low and condition index is high. A more thorough understanding of metal detoxification mechanisms will be necessary to help clarify the relationships between $[M]_{cyt}$/[MT] ratios, representing nonthionein metal binding or expressing different metal availabilities in the cytosol, and cellular and organism responses observed in this transplant experiment. In particular, the dynamics of Cd, Cu, and Zn in gill cytosols and gill tissues during the course of exposure should be studied not only in transplanted molluscs but also in the control groups. The binding of Cd to MT may be the result of a competition for binding sites between Cd and constitutive metals such as Cu and Zn (Bebianno et al. 1992; Brown et al. 1987).

(2) Lipid peroxidation and cellular Ca regulation processes have rarely been studied in situ in bivalve molluscs. The kinetics of cellular oxidative processes and effects of natural factors on these processes are poorly understood (e.g., Ribera et al. 1989). Moreover, free Ca^{2+} rather than total cytosolic Ca is carefully regulated by cells, because of the role of the former as an intracellular messenger (Carafoli 1987). Consequently, the total concentration of cytosolic Ca is less indicative of disturbed Ca regulation than would be the Ca^{2+} concentration. In addition, the model presented in Fig. 1 is oversimplified as it does not take into account the dynamics of reactive oxygen species in the cell.

(3) The model implicitly assumes that toxic effects should occur first at the biochemical level. Then, higher level effects might be expressed if regulatory mechanisms cannot compensate for chemical exposure. With the set of variables that we have used to evaluate the model, we have not been successful in demonstrating such a sequence of events.

Clearly, the hypothetical model of cytotoxicity that we have tested is oversimplified; the physiological and toxicological links between Cd, Cu, Ca, and MDA in *P. grandis* may well be more tenuous and complicated than described in Fig. 1. Refinement of the suggested cause–effect relationships must await further experimentation.

Relationships between metallothionein status and metal effects at higher levels of biological organization

Organism responses: transplanted bivalves

Although we have not unambiguously validated the hypothetical model of trace metal cytotoxicity (Fig. 1) in our field experiment, results related to this hypothetical chain of (cellular) events have been noted. Thus, a link may be seen to exist among the following phenomena: the appearance of nonthionein-bound Cd in the gill cytosol (as measured by HPLC), the oxidative degradation of membranes and disturbed cellular Ca concentrations. These cellular events are associated with a deterioration of the condition index of the transplanted bivalves (Fig. 2*a*). In addition to these responses, transplanted mussels grew more slowly than control mussels over the 400-d experiment.

These results are encouraging if we are to relate the degree of metal detoxification in *P. grandis* to the health status of the organism in its natural environment (criterion 5). Critical steps are a proper analytical separation of nonthionein-bound metal in cytosol preparation, and the determination of the dynamics of the key metals (Cd, Cu, and Zn) at the organ level. Also, a better understanding of the links between nonthionein-bound Cd and the organism responses would be desirable.

Responses at the population level

The apparent well being of the indigenous bivalves from Lake Vaudray (VA-IND), despite their chronic exposure to Cd and Cu, as well as the anticipated oxidative stress, contrasts with the precarious state of health of the transplanted bivalves. It is tempting to relate this apparent tolerance to the presence of high MT concentrations in individuals from the Lake Vaudray population. Moreover, because mining and smelting operations in the Rouyn-Noranda area date from 1927, Lake Vaudray bivalves might well have evolved a genetically based tolerance to trace metals, given the rapidity with which the evolution of metal tolerance can occur under high selection pressure (Bradshaw et al. 1990). Because of its genetic component, Luoma and Carter (1991) consider that elevated tolerance to a metal in one population, relative to other populations of the same species, might constitute a metal-specific response at the population level of organization. It follows that the extent of tolerance to trace metals as a result of selection, in populations of *P. grandis* in the Rouyn-Noranda area, is an important issue; it has to be resolved if we wish to use MT as an early warning indicator of metal exposure and stress in this species, or in any animal species in general (criterion 1). In other words, the questions are as follows:

(1) Provided that we have correctly accounted for the seasonal factors affecting MT levels in a given species at a given site (discussed in Couillard et al. 1995), can a high MT concentration under these conditions be considered an early signal of metal exposure and stress in this species? Or do high MT concentrations already constitute a response at the population level (a negation of the idea that [MT] could serve in an early warning capacity)?

(2) Will two populations of the same species, which differ in their MT genes, produce different MT levels under the same exposure conditions? If the answer is yes, then [MT] in a given species at a given site cannot be interpreted in its absolute sense; both genetic differences and differences in bioavailable metal concentrations will have to be considered to interpret the results correctly. Such limitations would restrict but not preclude the use of MT as a biomarker.

Table 5. Metallothionein status in the freshwater bivalve *Pyganodon grandis* as a potential biomarker of metal exposure and metal-induced stress. Summary of the results of the transplant experiment in relation to criteria for a biochemical indicator (see text for explanations).

Criteria No.	Results	Remarks
1. MT should have an early warning capacity; predictive of effects at higher levels of biological organization.	Our study: Possible link between [MT] and perturbations in reproduction in metal-impacted populations (preliminary results). See also Klaverkamp et al. (1991) and Johansson et al. (1986).	Long-term, field study is needed. Such a relation should also be sought in environments variously contaminated with trace metals. Relation between the MT response and genetic characteristics of the bivalve population should be addressed. Study of effects of trace metals on *P. grandis* larvae would be helpful.
2. MT should be specific to a particular trace metal or for a group of trace metals.	Our study: MT in *P. grandis* seems to respond strongly to Cd (Couillard et al. 1993, 1995).	Laboratory studies suggest that Cd is a good inducer of MT.
3. [MT] should respond in a concentration-dependent manner.	Our study: Strongly suggests such a response in an in situ situation whether on a temporal (Couillard et al. 1995) or spatial basis (Couillard et al. 1993).	Should be verified in a broader array of environments with varied physico-chemical conditions and with other trace metals.
4. All factors, endogenous as well as exogenous, affecting the MT should be known.	Our study: MT levels in our indigenous populations showed only modest seasonal fluctuations (encouraging) (Couillard et al. 1995).	Long-term, field study is needed. Seasonal fluctuations of [MT] in other environments with more complex hydrologic and geochemical processes should be studied.
5. Degree of metal detoxification should be related to the health status of the organism in its natural environment.	Our study: In gill cytosols of transplanted bivalves at 400 days, there is an apparent link among nonthionein-bound Cd, oxidative degradation of membranes, and disturbed cellular Ca concentration. The deterioration of condition indices suggests an occurrence of toxicity at the organism level (Preliminary results).	More thorough understanding of metal detoxification mechanisms in *P. grandis* is necessary, e.g., dynamics of Cd, Cu, and Zn in gill cytosols during long-term exposures.

Relationships between MT variations in response to metal exposure and population-level responses have also been examined in terms of the ability of the Lake Opasatica and Lake Vaudray bivalve populations to cope with their more or less metal-contaminated environments (notion of fitness). From the analysis of Tables 3 and 4, it would appear that developing larvae present in the gill marsupia of indigenous parent bivalves from Lake Vaudray, paradoxically a metal-tolerant population, face a Cd-induced stress. This stress might in turn result in reduced larval recruitment at the population level. Our analysis relies on similar molluscan stocks in similar environments using consistent sampling procedures. Nevertheless, unsuspected differences may exist. The impaired reproduction hypothesis should be tested concurrently in the field and in the laboratory under more controlled conditions.

Concluding remarks

The present field transplant experiment was designed to evaluate the potential of metallothionein as a biochemical indicator of prior metal exposure and metal-induced stress. Using as a framework the criteria recently proposed for an ideal biomarker (Stegeman et al. 1992; Haux and Förlin 1989), we have summarized the results of our experiment in Table 5 and identified areas where research is needed to refine this particular analysis. In the development of a validated biomarker for toxic metals, one of the crucial issues to be addressed is the relationship between the biomarker levels in a given population and the present or future state of the aquatic community.

The transplant experiment was designed to avoid ecological differences between sites, such that any changes in MT status and organism health could be attributed to a change in metal exposure. To minimize the influence of factors other than metal contamination on the biochemical and physiological responses considered, we selected sites that shared similar ecological characteristics. Similarities in the availability of resources for our test species were assessed by comparing the condition indices and growth rates of the respective indigenous bivalve populations. Such approaches have been used by others (McCuaig and Green 1983; Haukioja and Hakala 1978). The rigorous verification of this assumption of ecological similarity would ultimately require intensive temporal sampling, simultaneously at the two sites, of target variables reflecting the availability of resources and habitat quality, e.g., the suspended food particle size spectrum, calorific values for

these food particles, degree-days, water turbulence, wind exposure, etc.

Although not yet at an application stage, MT monitoring in *P. grandis* would benefit from the qualities of molluscs as bioindicators of trace metal pollution (Phillips 1980). In particular, *P. grandis* is able to tolerate very high contaminant burdens and survive without undue adverse effects. As a sedentary organism it is representative of the area of collection. It is long lived, of reasonable size, and is widely disseminated in North America (Clarke 1981). It is also easy to transplant; transplantation of specimens with similar contamination histories and MT genetic characteristics could provide strong evidence for site-specific impacts (Clements 1991). Such an approach would be well suited for the assessment of environmental benefits gained by pollution control measures.

One of the main problems impeding the field use of biochemical indicators of contaminant effects is that research attention has focused on developing new methods, rather than on improving and validating known biochemical responses for field use (Veersteg et al. 1988). In the development of biomonitors, determinations of no observable effect concentrations (NOEC) are often subject to regulatory considerations (the question being whether metals at measured environmental levels have a demonstrable biological effect). Changing the focus to a scientific goal of understanding how metals affect biological responses might be more profitable (Luoma and Carter 1991).

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Sampling design and statistical considerations for the commercial groundfish fishery of Oregon

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Abstract: Fisheries management is often based on data collected through various sample survey programs. At a minimum, commercial fisheries must be monitored to determine the species and age compositions of the landings; this provides the baseline data on which to assess the stocks. An equally important objective, which is often ignored, is the determination of the variability associated with derived estimates. This paper presents measures of dispersion for landing estimates generated from a two-stage sampling design used for commercially harvested groundfish species landed at Oregon ports; particular attention is given to the relative magnitudes of variability at the first and second stages of sampling designs used for estimating species and age compositions. At least two-thirds of the total amount of rockfish landed in each port/quarter stratum consisted of estimates of species composition that had small coefficients of variation (CV <10%). For each species sampled for age composition, at least three-fourths of the total landings included estimates for individual ages with CVs <25%. For the majority of the landings, the variation at the first stage of sampling contributed at least 63 and 90% to the variance of the estimates for the species and age compositions, respectively.

Résumé : La gestion des ressources halieutiques s'appuie souvent sur les données recueillies par divers programmes d'inventaires. Il convient, au minimum, de contrôler les prises commerciales au débarquement afin d'en déterminer la composition (espèces et classes d'âge) aux fins de l'évaluation des stocks. Un autre objectif important, mais souvent ignoré, consiste à déterminer la variabilité des estimations ainsi obtenues. Le présent article expose des mesures de la dispersion des estimations obtenues à l'aide d'un protocole d'échantillonnage à deux volets utilisé pour les prises commerciales de poissons de fond débarquées dans les ports de l'Oregon. On s'attache en particulier à déterminer l'amplitude relative de la variabilité à la première et à la seconde étape de l'échantillonnage destiné à fournir une estimation de la composition par espèces et par groupes d'âge. Les deux tiers au moins du volume total de sébastes débarqués pour chaque tranche « port/trimestre » présentait des compositions par espèces estimatives assorties de petits coefficients de variation (CV < 10%). Pour chaque espèce dont on a déterminé la répartition par classes d'âge, les trois quarts au moins du total des débarquements incluaient des estimations de l'âge de spécimens individuels dont le CV était inférieur à 25%. Pour la majorité des débarquements, les variations intervenues lors de la première étape de l'échantillonnage étaient responsables d'au moins 63 et 90% de la variance des estimations de la composition par espèces et par groupes d'âge respectivement.

[Traduit par la Rédaction]

Introduction

The basic types of data usually regarded as necessary for current stock assessments are estimates of the species and age compositions of landings, fishing effort, and various demographic characteristics of the exploited fish populations, such as sex ratios, maturity schedules, and length distributions. To obtain these data, fishery management agencies most often monitor the commercial landings by utilizing appropriate sampling designs, which require the selection of a smaller component of a larger target population. For example, inferences about the total landings of groundfish in a specified time period (i.e., the population) can be made from information contained in a random sample of the boat-trip landings that constituted the population.

Popular stock assessment models, such as virtual population analysis (Gulland 1965), cohort analysis (Pope 1983), and stock synthesis analysis (Methot 1990) use estimates of landings and age compositions to determine the pattern of historical abundance and derive catch quotas for exploited species. The conclusions drawn from stock assessments rely largely on the reliability associated with the sampled landing data (Pope and Gray 1983; Beddington et al. 1984; Shepherd 1988; McAllister and Peterman 1992).

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Without estimates of the variability of the landing data, stock assessment teams must utilize input data that, at the very best, are subject to increased uncertainty and, at the worst, generate misleading results. As stock assessment techniques continue to gain acceptance in broad areas of fishery science, it becomes imperative to examine rigorously the relationship between successful management approaches and the precision and accuracy of the sampling data (Larkin 1972; Powles 1983; Pope 1988; Pelletier and Gros 1991).

In an ideal setting, the sampling of commercial landings is a routine task and appropriate designs, field protocols, and estimation procedures have been discussed in varying detail (Gulland 1955, 1966; Tomlinson 1971; Bazigos 1974; Quinn et al. 1983; Sen 1986; Pope 1988). However, in most cases, management programs are circumscribed by financial and logistical constraints, which dictate sampling methods that provide estimates of population totals, averages, and proportions, while at the same time reduce the size of the sampling operations. Additionally, the unique characteristics of many shoreside processing facilities, where sampling occurs, dictate that data collection procedures be flexible. The statistical properties of current sampling designs, particularly the precision of the landing estimates, need to be documented before alternative field and estimation techniques can be objectively evaluated.

This study addressed sampling designs and statistical considerations for the commercial groundfish fishery in Oregon. The primary objective of the study was to evaluate the statistical performance of a two-stage sampling design developed by Sen (1986). In this paper, I document the sampling variability associated with the species and age compositions of groundfish landings in Oregon. Particular attention is given to the relative magnitudes of variance at the first and second stages of the sampling designs, namely the variability among primary units (boat trips) versus variability among secondary units (baskets of fish), within boat trips. Additionally, the landing statistics presented here are used to discuss the appropriateness of a sampling design, which is currently under consideration, that requires selecting only a single basket of fish within each boat trip.

Methods

In 1989 the Oregon Department of Fish and Wildlife adopted sampling methods proposed by Sen (1986), who documented effective techniques for sampling groundfish species landed at California ports. Generally speaking, the field protocols for sampling groundfish in Oregon closely follow the data collection procedures used by the California Department of Fish and Game.

Groundfish landings in Oregon are primarily sampled to obtain estimates of species and age compositions. Sampling for species composition is required because boat-trip landings contain a mix of species that are unloaded by sort groups (market categories) rather than by individual species. In general, sort groups are determined by market demands, as well as by edicts from the Oregon Department of Fish and Wildlife. In most cases, market categories contain more than one species and the types and amount of each species contained in a market category varies among boat trips. The common and scientific names for the species of groundfish discussed in this paper are presented in Table 1.

Table 1. Common and scientific names for groundfish species (Robins et al. 1991).

Common name	Scientific name
Rougheye rockfish	*Sebastes aleutianus*
Pacific ocean perch	*Sebastes alutus*
Aurora rockfish	*Sebastes aurora*
Redbanded rockfish	*Sebastes babcocki*
Shortraker rockfish	*Sebastes borealis*
Silvergrey rockfish	*Sebastes brevispinus*
Greenspotted rockfish	*Sebastes chlorostictus*
Darkblotched rockfish	*Sebastes crameri*
Splitnose rockfish	*Sebastes diploproa*
Greenstriped rockfish	*Sebastes elongatus*
Widow rockfish	*Sebastes entomelas*
Yellowtail rockfish	*Sebastes flavidus*
Chilipepper	*Sebastes goodei*
Rosethorn rockfish	*Sebastes helvomaculatus*
Shortbelly rockfish	*Sebastes jordani*
Cowcod	*Sebastes levis*
Black rockfish	*Sebastes melanops*
Blackgill rockfish	*Sebastes melanostomus*
Tiger rockfish	*Sebastes nigrocinctus*
Speckled rockfish	*Sebastes ovalis*
Bocaccio	*Sebastes paucispinus*
Canary rockfish	*Sebastes pinniger*
Redstripe rockfish	*Sebastes proriger*
Yellowmouth rockfish	*Sebastes reedi*
Yelloweye rockfish	*Sebastes ruberrimus*
Bank rockfish	*Sebastes rufus*
Stripetail rockfish	*Sebastes saxicola*
Pygmy rockfish	*Sebastes wilsoni*
Sharpchin rockfish	*Sebastes zacentrus*
Shortspine thornyhead	*Sebastolobus alascanus*
Longspine thornyhead	*Sebastolobus altivelis*
English sole	*Parophrys vetulus*
Dover sole	*Microstomus pacificus*

In this paper a landing is defined as the entire amount of fish, expressed in weight or number, brought ashore by a single boat that has completed a fishing trip. The population of interest depended on the sampling objective. For species-composition sampling, the population was the total amount of rockfish harvested by trawling gears and landed at Oregon ports during the study period. For age-composition sampling, the population was the total amount of a particular species (selected rockfish and flatfish) harvested by trawling gears and landed at Oregon ports during the study period. An important feature of the sampling design was that landing estimates were based on market categories, rather than the boat trips themselves. That is, a landing was subdivided into the market categories it contained and sampling took place at the market-category level. Ultimately, the objectives of sampling were to determine (*i*) species compositions of the market categories or (*ii*) age compositions of species of interest, which were selected from particular market

categories. Methods are presented separately for the species- and age-composition sampling programs.

Species-composition sampling program

The analyses of species composition for this study were based on a two-stage random sampling plan combined with stratification. Port and quarter (a year partitioned into four 3-month blocks) combinations were treated as strata and boat trips within a stratum as primary units; primary units were selected at the first stage of the design. The boat-trip landings were poststratified into market categories. At least two baskets of fish (secondary units) of a fixed weight were subsampled within each market category; the secondary units were selected at the second stage of the design.

Currently, more than 20 ports along the Oregon coastline process commercially landed finfish; however, three of these ports receive nearly all of the landings and these sites are the most heavily sampled. Sampling duties are the responsibility of port biologists (samplers) assigned to the three ports: Astoria, Newport, and Coos Bay.

Field sampling procedures

The following discussion is a summary of the sampling protocols used by the port biologists. Rockfish landings were sampled for species composition. The majority of the rockfish were landed in six market categories: (*i*) widow rockfish (WDW), (*ii*) yellowtail rockfish (YWT), (*iii*) Pacific ocean perch (POP), (*iv*) thornyhead (TYH), (*v*) large rockfish complex (LRC), and (*vi*) small rockfish complex (SRC). Henceforth, the three-letter acronyms are used to distinguish the market categories from the species contained within them. A boat trip could include any combination of the six market categories. Port biologists most often sampled only one market category per boat trip. However, in some cases, two and even three market categories were sampled per trip.

In general, for each market category, port biologists were instructed to obtain five boat-trip samples per 100 t of the category landed. That is, each sample consisted of two to six baskets of fish (secondary units) selected from a market category (poststratification unit) within a boat trip (primary unit). Sampling protocols required at the second stage of the design were as follows: (*i*) two to four 11.34-kg (25 lb) baskets were taken for the WDW and TYH market categories; (*ii*) two to four 22.68-kg (50 lb) baskets were selected for the YWT and POP market categories; (*iii*) four to six 11.34-kg baskets were chosen for the SRC market category; and (*iv*) four to six 22.68-kg baskets were selected for the LRC market category. Landings were originally weighed in English units (lb) and these data were then converted to metric units (kg) for all analytical procedures.

At the processing facilities, the fish were removed from the hulls of the vessels and placed in totes (approximately 360-kg capacity plastic, metal, or wooden bins). The totes were then either immediately transported to processing rooms (via forklifts, conveyor belts, or vacuums) or placed in a temporary cold storage room within the facility. The biologists sampled the totes while they were en route to the processing rooms, usually as the vessel was being unloaded, or while the totes were in cold storage. No single dockside sampling technique was ideal for all processing facilities. The samplers were instructed to select baskets of fish from totes separated over the entire unloading time of a vessel, e.g., a basket of fish from one of the first totes unloaded and a basket from later in the unloading operation. The individual fish selected for each basket were taken from one corner of a tote, starting at the top and working to the bottom, trying not to account consciously for sizes or species of fish selected. The samplers recorded the aggregate weights for each species contained in a basket.

Age-composition sampling program

The sampling design used to collect age-composition data was similar to the design presented above for species-composition sampling, but the two designs were not identical in all respects. The following discussion addresses sampling procedures unique to the age-composition sampling program.

During the study period, only landings of widow rockfish, yellowtail rockfish, canary rockfish, English sole, and Dover sole were routinely sampled for age composition. Because smaller sample sizes were allocated to the age-composition sampling program than to the species-composition sampling program, only Oregon coastwide estimates of age composition were calculated.

Sampling for age composition required selecting specimens only from certain market categories. For example, age-composition samples for yellowtail rockfish required selecting only yellowtails from the YWT market categories, and these fish were assumed to constitute random samples from the entire landings of yellowtail rockfish. This selection approach was adopted because the species involved in the age-composition sampling program were most often landed within a single market category, which was usually their own. Canary rockfish lack a market category of their own and specimens of this species were sampled only from the LRC market category, which was the category in which these rockfish were most often landed.

For each market category, port biologists were instructed to obtain two boat-trip samples per 100 t of the category landed. Biologists most often selected two baskets of fish, of fixed weight, within each sampled market category. For widow rockfish, English sole (ENG), and Dover sole (DOV), 11.34-kg baskets were selected, and for yellowtail rockfish and canary rockfish, 22.68-kg baskets were taken. Age structures, interopercles for English sole and otoliths for all other species, were removed from the sampled fish, placed in storage vials, and examined for age determination at a later date.

Estimation procedures

The formulae for estimators of mean and total landings and their errors documented by Sen (1984, 1986) were generally applicable to the Oregon fishery data. The formulae presented in Sen (1986) that were used in this study are relatively straightforward statistical methods used in sampling (e.g., Sukhatme and Sukhatme 1970; Cochran 1977; Scheaffer et al. 1990), which have been applied to commercial fishery data.

The statistical framework developed by Sen (1984, 1986) allowed estimation procedures to be applied similarly to samples of both species and age compositions. For a species-composition sample, the measurement variable is the

weight of a particular species in a basket selected from a market category contained within a boat trip. For an age-composition sample, the measurement variable is the number of fish of a particular age in a basket selected from a market category contained within a boat trip. All notation and formulae used to derive landing statistics presented here are given in the Appendix.

The analyses of age composition for the rockfish species (widow, yellowtail, and canary rockfish) required that the market categories (WDW, YWT, and LRC) be sampled first for species composition so that the total landings of each market category, which were used as weighting variables in the estimation formulae, were appropriately adjusted to reflect the contribution of the species sampled. The ENG and DOV market categories were assumed to contain only English sole and Dover sole, respectively; thus, these categories were not sampled for species composition.

By using standard two-stage variance expressions (Cochran 1977; Scheaffer et al. 1990), the variance associated with the landing estimates was partitioned into between ($\hat{V}_{BTW}$ %) and within ($\hat{V}_{WTH}$ %) boat-trip components, which reflected variation percentages at the first and second stage of the design, respectively. Estimates of species composition and their variances were determined within market categories for each stratum (port/quarter) and then summed across market categories for calculating totals within strata. As stated previously, only Oregon coastwide estimates were generated for species included in the age-composition sampling program. I use the CV as the measure of dispersion associated with a landing estimate. The CV was calculated as [(standard error of the landing estimate/total landing estimate)100]; this statistic is also referred to as a relative standard error (Som 1973) and a coefficient of variation of the estimate (Cochran 1977).

Results

Results are presented separately for the species- and age-composition sampling programs. Estimates of species composition are presented as weight (kg) of fish landed and estimates of age composition are presented as numbers of fish landed.

Species-composition sampling program

Species-composition data collected from August 1991 through March 1992 were analyzed in this study. Results from analyses of completely sampled quarters are presented here, namely the fourth quarter (October–December) 1991 and first quarter (January–March) 1992. Results are based on analyses of six port/quarter strata; two quarters for each of the three ports. General patterns for landing estimates and their errors that existed across the strata are presented. Additionally, results for Newport in the first quarter 1992 are used to illustrate the statistical properties of the rockfish landings for a typical and complete stratum.

The LRC market category in each stratum always contained only a single species, canary rockfish, that composed greater than 25% of the total landings. For example, in a stratum, canary rockfish never constituted less than 35 012 kg or more than 193 890 kg and never less than 28% or more than 93% of the LRC market category (Table 2). The CV of the estimates for canary rockfish landed in the LRC market category ranged from 8 to 41%; however, most often these landing estimates for canary rockfish were relatively precise, with CVs <15%. The remaining species composition for the LRC market category in each stratum included from 6 to 20 species that individually contributed less than 18% to the total landings and the CV of the landing estimates for these species ranged from 20 to 143% (Table 2); however, most often these landing estimates were highly variable (CVs >50%).

The SRC market category in each of the six strata always contained one or two species (yellowmouth and (or) darkblotched rockfish) that composed greater than 25% of the total landings (Table 2). The CV of the landing estimates were fairly large (22–35%) for species that contributed greater than 25% to the total landings of the SRC market category. In cases (strata) where darkblotched and yellowmouth rockfish individually composed less than one-fourth of the total landings of the SRC market category, the landing estimates were highly variable, with CVs that ranged from 58 to 73%. The species composition of the SRC market category in each stratum contained from 16 to 18 additional rockfish species that individually composed less than 20% of the total landings and had estimates that were highly variable, where CVs ranged from 23 to 121%, with the majority greater than 40%.

The TYH market category in each stratum always consisted only of shortspine and longspine thornyhead (Table 2). The estimates for thornyhead species landed in the TYH market category were very precise (CVs <15%) in cases (four of the six strata) where these species composed at least one-third of the total landings.

The POP, YWT, and WDW market categories contained primarily their own species, namely Pacific ocean perch, yellowtail rockfish, and widow rockfish, for the three market categories, respectively (Table 2). The landing estimates for these species within their respective market categories were very precise, with CVs always ≤1%. For two of the six strata, the YWT and WDW market categories contained only their own species (yellowtail and widow rockfish, respectively), which resulted in CVs = 0%. In cases where the POP, YWT, and WDW market categories did include additional rockfish, from one to five species individually composed less than 1% of the total landings and had highly variable landing estimates, with CVs that ranged from 54 to 113%.

In general, most of the variability associated with landing estimates for individual species was due to the among boat-trip component of variation ($\hat{V}_{BTW}$ %); however, the magnitude of $\hat{V}_{BTW}$ % was not consistent across all of the market categories. That is, the variation within boat trips ($\hat{V}_{WTH}$ %) was large enough to warrant consideration in some situations. With the exception of the species landed in the SRC market category, $\hat{V}_{BTW}$ % composed at least 63% of the estimated variance of the landing estimates for the individual species (Fig. 1). The SRC market category was the only category in which landing estimates were characterized by substantial amounts of second-stage sampling error ($\hat{V}_{WTH}$ %). In general, $\hat{V}_{BTW}$ % was the highest and most consistent (i.e., generated narrow ranges) for species landed in the LRC, TYH, and WDW market categories, where at least 80% of the estimated variance associated with these landing estimates

Table 2. Species-composition summaries by market category for rockfish landings in Oregon, October 1991 – March 1992. Ranges for landing estimates (kg), percent of market-category total landings, and CV are based on species-composition results for market categories within six port/quarter strata. For each market category, rockfish species are listed in descending order according to maximum percent contribution to market-category total landings.

		Range[a]		
Market category[b]	Rockfish species	Landing estimate ($\hat{Y}_{ij}$)	Percent of market-category total landings	CV (%)
LRC	Canary	35 012 – 193 890	28 – 93	8 – 41
	Bocaccio	0 – 35 960	0 – 18	25 – 84
	Darkblotched	2 413 – 26 127	2 – 18	40 – 123
	Shortraker	0 – 39 216	0 – 15	49 – 69
	Yellowmouth	0 – 23 125	0 – 12	27 – 58
	Rougheye	235 – 30 024	<1 – 12	20 – 120
	Yelloweye	0 – 9 627	0 – 11	42 – 119
	Splitnose	0 – 9 242	0 – 7	44 – 82
	Redstripe	0 – 8 450	0 – 4	49 – 83
	Chilipepper[c]	0 – 3 450	0 – 4	103
	Bank	0 – 6 873	0 – 3	71 – 88
	Redbanded	0 – 8 426	0 – 3	64 – 110
	Sharpchin	0 – 7 862	0 – 3	44 – 70
	Silvergrey	74 – 4 179	<1 – 3	37 – 113
	Aurora	0 – 5 333	0 – 2	57 – 104
	Pacific ocean perch	500 – 3 557	<1 – 2	51 – 118
	Greenstriped	0 – 2 810	0 – 1	50 – 100
	Miscellaneous[d]	38 – 1 325	<1	54 – 143
SRC	Yellowmouth	0 – 119 041	0 – 63	22 – 73
	Darkblotched	13 521 – 37 690	20 – 47	22 – 58
	Redstripe	177 – 8 943	<1 – 20	30 – 101
	Sharpchin	246 – 14 953	<1 – 20	39 – 69
	Greenstriped	172 – 5 584	<1 – 13	40 – 117
	Pacific ocean perch	2 502 – 6 241	3 – 8	23 – 47
	Canary	0 – 4 055	0 – 8	28 – 110
	Splitnose	823 – 5 400	1 – 8	34 – 42
	Rougheye	116 – 7 642	<1 – 5	25 – 109
	Yelloweye	0 – 2 163	0 – 2	46 – 87
	Bocaccio	0 – 1 105	0 – 2	58 – 116
	Bank	0 – 2 971	0 – 2	48 – 102
	Redbanded	0 – 1 189	0 – 2	60 – 121
	Aurora	89 – 2 769	<1 – 1	27 – 102
	Rosethorn	35 – 537	<1 – 1	57 – 70
	Miscellaneous[d]	9 – 551	<1	46 – 116
TYH	Shortspine thornyhead	15 820 – 269 053	9 – 92	7 – 61
	Longspine thornyhead	23 429 – 287 594	8 – 91	6 – 58
POP	Pacific ocean perch	16 883 – 28 276	98 – 99	<1 – 1
	Miscellaneous[d]	9 – 253	<1	54 – 113
YWT	Yellowtail	47 388 – 580 767	99 – 100	0 – 1
	Miscellaneous[d]	24 – 1 208	<1	64 – 108

Table 2 (*concluded*).

Market category[b]	Rockfish species	Range[a] Landing estimate ($\hat{Y}_j$)	Percent of market-category total landings	CV (%)
WDW	Widow	40 064 – 516 605	99–100	0–1
	Miscellaneous[d]	581 – 2 459	<1	60–102

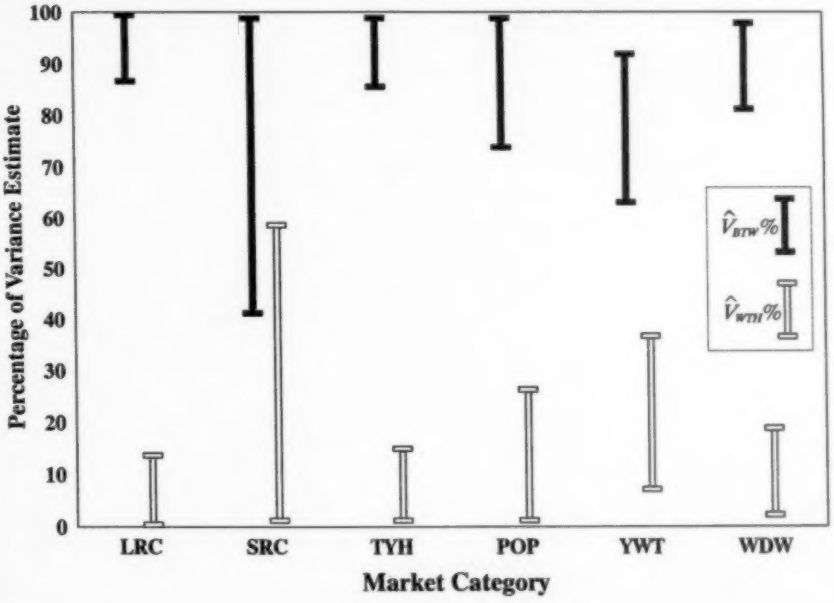
[a]Ranges for landing estimates and percent of market-category total landings that include zero indicate the rockfish species was not landed in the noted market category of all six strata. Ranges for CV are based on market categories of strata that rockfish species were landed in.

[b]Market-category acronyms are as follows: LRC is large rockfish complex, SRC is small rockfish complex, TYH is thornyhead, POP is Pacific ocean perch, YWT is yellowtail rockfish, and WDW is widow rockfish.

[c]Chilipepper was landed in the LRC market category of only one stratum, thus, a single CV estimate is presented.

[d]Miscellaneous includes rockfish species that composed <1% of the total landings (in weight) of a market category in at least one stratum: 6 species in LRC, 12 in SRC, 4 in POP, 6 in YWT, and 2 in WDW.

Fig. 1. Ranges for between ($\hat{V}_{BTW}$ %) and within ($\hat{V}_{WTH}$ %) boat-trip components of variation, expressed as percentages of the total variance estimates, associated with rockfish landings in six market categories across six port/quarter strata (October 1991 – March 1992). Only landing estimates that had CVs >1% are included in the ranges. Market-category acronyms are as follows: LRC is large rockfish complex, SRC is small rockfish complex, TYH is thornyhead, POP is Pacific ocean perch, YWT is yellowtail rockfish, and WDW is widow rockfish.



was due to the variation at the first stage of sampling. In general, the POP and YWT market categories were characterized by landing estimates with $\hat{V}_{BTW}$ % values that were slightly lower and less consistent (i.e., generated wider ranges) than those observed for species landed in the LRC, TYH, and WDW market categories.

In each stratum, at least two-thirds of the total landings of rockfish consisted of from three to five species that had very precise landing estimates, with CVs <10%. There were six species that were estimated with high precision (CV <10%) in at least one stratum (Table 3). With the exception of canary rockfish, which was primarily landed in the LRC market category, all of the species that had precise estimates were primarily landed in their own market categories. The remaining species composition of each stratum included rockfish that were landed mostly in the LRC and SRC market categories and these landing estimates were highly variable, with CVs that ranged from 20 to

Table 3. Species-composition summary for total rockfish landings in Oregon, October 1991 – March 1992. Ranges for landing estimates (kg), percent of stratum total landings, and CV are based on species-composition results for six port/quarter strata. Rockfish species are listed in descending order according to maximum percent contribution to stratum total landings.

	Range[a]		
Rockfish species	Landing estimate ($\hat{Y}_{..}$)	Percent of stratum total landings	CV (%)
Yellowtail	47 388 – 584 122	5–38	0–1
Widow	40 144 – 517 813	4–34	0–1
Shortspine thornyhead	15 830 – 269 147	1–34	7–61
Longspine thornyhead	23 429 – 287 594	3–31	6–58
Canary	65 774 – 194 009	7–21	7–13
Yellowmouth	0 – 139 901	0–18	20–66
Darkblotched	15 934 – 63 816	2–8	20–39
Pacific ocean perch	3 002 – 34 784	<1–4	5–44
Bocaccio	0 – 37 011	0–2	25–84
Rougheye	351 – 16 679	<1–2	42–105
Sharpchin	1 677 – 14 953	<1–2	43–65
Redstripe	1 483 – 10 014	<1–1	46–69
Splitnose	857 – 9 076	<1–1	35–49
Miscellaneous[b]	15 – 10 685	<1	27–121

Note: Stratum landing estimates for rockfish species were calculated by simply summing market-category estimates within strata (see Estimation procedures and Appendix).

[a]Ranges for landing estimates and percent of stratum total landings that include zero indicate the rockfish species was not landed in all six strata. Ranges for CV are based on strata that rockfish species were landed in.

[b]Miscellaneous includes 15 rockfish species that always composed <1% (in weight) of the total landings of a stratum.

121% (Table 3), the majority of which were greater than 40%. For example, yellowtail rockfish, which was primarily landed in the YWT market category, composed from 5 to 38% of the total landings in each stratum and was always estimated with very high precision, whereas darkblotched rockfish, which was primarily landed in the LRC and SRC market categories, composed from 2 to 8% of the total landings in each stratum and was never measured with high precision (Table 3).

The estimates for Newport in the first quarter of 1992 (Table 4) were typical of the general characteristics of species composition presented above (Tables 2 and 3, Fig. 1). The LRC and SRC market categories consisted primarily of species that were not estimated with high precision. Canary rockfish, which composed over one-half of the LRC market category, was the only species landed in either of the complex market categories that had a CV of the landing estimate less than 15%.

The TYH, POP, YWT, and WDW market categories consisted primarily of their own species, namely thornyhead species, Pacific ocean perch, yellowtail rockfish, and widow rockfish, for the four market categories, respectively (Table 4). With the exception of shortspine thornyhead, which composed less than 10% of the TYH market category, all of the above species were estimated with very high precision, with CVs <10%. The other rockfish landed in these four market categories had estimates that were highly variable, with CVs >55%.

For Newport in the first quarter of 1992, at least 70% of the variance associated with the estimated landings for species in the LRC, TYH, POP, YWT, and WDW market categories was due to variation among boat trips, $\hat{V}_{BTW}$ %, (Table 4). The estimated variances associated with landings of darkblotched and yellowmouth rockfish in the SRC market category, which together composed roughly 70% of the total landings, were also primarily due to $\hat{V}_{BTW}$ %; however, the remaining 30% of the species composition included landing estimates with variances that incorporated considerable amounts of second-stage sampling error ($\hat{V}_{WTH}$ %).

Approximately 90% of the total landings for Newport in the first quarter of 1992 included species that had precise landing estimates, with CVs ≤12% (Table 5). With the exception of canary rockfish, which was primarily landed in the LRC market category (Table 4), the species that composed the precise landing information were landed in their own market categories. The remaining 14 species were primarily landed in the LRC and SRC market categories and had landing estimates that were highly variable, where CVs ranged from 20 to 83%, the majority of which were greater than 40%.

Age-composition sampling program

Age-composition data collected from January to December 1991 were analyzed for this study. In general, age-composition statistics were very consistent for all five species analyzed. Additionally, results from analyses of yellowtail rockfish are

presented to highlight the general discussion and illustrate the statistical properties of a typical and complete age composition of a species.

For the most part, results from age-composition analyses were more variable, albeit slightly, than landing estimates of species composition. That is, the percentage of the age composition of each species that included landing estimates with CVs <10% was smaller than the percentage of the total landings of each stratum that consisted of precise estimates of species composition.

All five species were characterized by a relatively small range of consecutive ages that together composed the majority of the total landings (Table 6). At least 89% of the total landings of each species included ages that individually contributed greater than 1% to the total landings and, for the most part, these estimates were relatively precise, with CVs ≤25%. The remaining 4–11% of each age composition consisted of 9 to 28 ages that individually contributed ≤1% to the total landings, with from one to three younger ages preceding and from 8 to 25 older ages following the group of ages that constituted the majority. In general, for all species, highly variable landing estimates characterized the ages that constituted ≤1% of the total landings, with CVs usually greater than 40%.

For each species, landing estimates for individual ages had estimated variances that were almost due entirely to variation among boat trips ($\hat{V}_{BTW}$ %). For all five species analyzed, at least 90% of the variance of the landing estimate for each age in the composition was due to V_{BTW} %.

Estimates of age composition for yellowtail rockfish landed at Oregon ports in 1991 were typical of the general age-composition results (Table 7). Fish from ages 6 to 17 composed roughly 94% (1 245 053 fish) of the total landings of yellowtail rockfish and the landing estimates for the individual ages were relatively precise, with CVs ≤25%. The remaining approximately 6% of the age composition included 21 ages that individually did not contribute more than 1% to the total landings and these estimates were generally much more variable than those ages associated with the 94% majority. At least 96% of the variance of the landing estimate for each age was due to differences between the sampled boat trips at the first stage of sampling ($\hat{V}_{BTW}$ %).

Discussion

The sampling designs used in this research provided effective methods for sampling groundfish landings in Oregon. In general, the designs generated relatively precise results; however, the statistical properties of the landing estimates were not identical between the species- and age-composition sampling programs, which in effect, produced different conclusions in some cases. The most important difference between the results generated from the two analyses was the magnitude and consistency of the variation at the second stage of sampling ($\hat{V}_{WTH}$ %). The impact of the variability at the second stage of sampling is of particular importance presently, because time and financial constraints have caused Oregon and California groundfish management to consider less rigorous sampling protocols than those currently in use. Investigations regarding the benefits and costs of selecting only a single basket of fish within each sampled boat trip have been proposed as research areas that need to be addressed so that revisions to current designs can be evaluated appropriately (U.S. Pacific Coast Groundfish Statistics Working Group, D.B. Sampson (Chairperson), Oregon State University, Hatfield Marine Science Center, Newport, OR 97365, personal communication).

Considerable amounts of second-stage variation characterized substantial portions of the species-composition results. In particular, the species that contributed small percentages (<1%) to the total landings of each market category and many of the species landed in the SRC market category had estimated variances that were due, in large part, to sampling error at the second stage of the design. The species-composition results clearly indicate that a modified version of the current design, one that requires only a single basket of fish at the second stage, will produce seriously biased variance estimates in many cases. It may be valid in some cases to assume that the variation within a boat trip ($\hat{V}_{WTH}$ %) is insignificant and does not warrant sampling consideration; however, where and when this is true cannot be predicted. The results presented here indicate that selecting two baskets of fish from a market category within a boat trip would provide relatively reliable and accurate sampling information for the majority of the rockfish landings, and that taking more than two baskets of fish will have little influence on the final variance estimates. If selecting and recording a second basket causes a port biologist to forgo sampling other boat trips, then a trade-off between accuracy and precision of the landing statistics may need further evaluation.

Results from age-composition analyses provide some evidence that an abbreviated version of the complete two-stage sampling approach, one based on a single basket of fish at the second stage, could be used without compromising the validity of the landing estimates. For each species in the age-composition sampling program, at least 90% of the variance associated with the landing estimates for individual ages was due to variation among boat trips ($\hat{V}_{BTW}$ %) and very little variation existed among baskets of fish selected within each boat trip ($\hat{V}_{WTH}$ %). Although selection of single baskets of fish from the sampled boat trips would result in biased variance estimates, the bias may not be important for all practical purposes. This finding is likely to be of considerable benefit to this, as well as other age-composition sampling programs, given the technical difficulties and additional time involved in removing age structures from the individual fish.

It should be emphasized that whenever possible a second basket of fish should be selected to ensure unbiased variance estimates. If it is decided that the selection of a single basket of fish at the second stage of the design is appropriate, then I recommend that a short-term, complete two-stage design be used periodically to validate that $\hat{V}_{WTH}$ % is inconsequential for management concerns.

In general, variance estimates associated with landing estimates from the species- and age-composition sampling programs included relatively small amounts of second-stage variation; therefore, a maximum of two baskets of fish should be selected within each sampled market category. The only possible exception to this recommendation

Table 4. Species-composition estimates by market category for the Newport/first quarter 1992 stratum. Landing estimates are in kilograms of fish. Between ($\hat{V}_{BTW}$ %) and within ($\hat{V}_{WTH}$ %) boat-trip components of variation are expressed as percentages of the estimated total variance of the landing estimates.

Market category[a]	Rockfish species	Landing estimate ($\hat{Y}_{.j}$)	Percent of market-category total landings	$\hat{V}_{BTW}$ %	$\hat{V}_{WTH}$ %	CV (%)
LRC						
(n_j = 13)[b]	Canary	103 264	52	94	6	12
	Bocaccio	35 960	18	97	3	25
	Yellowmouth	23 125	12	98	2	58
	Yelloweye	9 627	5	98	2	51
	Redstripe	8 450	4	99	1	53
	Bank	6 873	3	90	10	88
	Darkblotched	3 093	2	96	4	46
	Silvergrey	2 460	1	87	13	62
	Pacific ocean perch	2 111	1	92	8	77
	Rougheye	1 295	<1	97	3	104
	Splitnose	1 107	<1	99	1	82
	Sharpchin	1 041	<1	93	7	58
	Yellowtail	735	<1	86	14	57
	Greenstriped	228	<1	100	0	100
	Rosethorn	44	<1	88	12	86
	Subtotal	199 413	100			
SRC						
(n_j = 13)	Darkblotched	20 241	38	97	3	22
	Yellowmouth	17 353	33	97	3	26
	Canary	4 055	8	68	32	28
	Pacific ocean perch	2 646	5	83	17	24
	Rougheye	2 373	5	41	59	32
	Greenstriped	1 146	2	94	6	55
	Yelloweye	1 058	2	79	21	46
	Bocaccio	1 051	2	64	36	58
	Splitnose	823	2	70	30	34
	Redstripe	733	1	72	28	30
	Silvergrey	519	<1	54	46	46
	Bank	406	<1	64	36	48
	Sharpchin	246	<1	74	26	39
	Aurora	219	<1	74	26	47
	Yellowtail	161	<1	57	43	86
	Rosethorn	35	<1	43	57	70
	Shortspine thornyhead	31	<1	43	57	100
	Subtotal	53 096	100			
TYH						
(n_j = 5)	Longspine thornyhead	158 805	91	99	1	6
	Shortspine thornyhead	15 820	9	99	1	61
	Subtotal	174 625	100			
POP						
(n_j = 5)	Pacific ocean perch	16 883	99	73	27	1
	Aurora	121	<1	75	25	56
	Yellowmouth	61	<1	90	10	82
	Splitnose	9	<1	92	8	113
	Subtotal	17 074	100			
YWT						
(n_j = 30)	Yellowtail	580 767	>99	75	25	1
	Widow	1 208	<1	86	14	64
	Redstripe	503	<1	70	30	74
	Sharpchin	390	<1	70	30	99
	Subtotal	582 868	100			

Table 4 (*concluded*).

Market category[a]	Rockfish species	Landing estimate ($\hat{Y}_j$)	Percent of market-category total landings	$\hat{V}_{BTW}$ %	$\hat{V}_{WTH}$ %	CV (%)
WDW						
(n_j = 38)	Widow	516 605	>99	85	15	1
	Yellowtail	2 459	<1	88	12	60
	Subtotal	519 064	100			
	Total	1 546 140				

[a]Market-category acronyms are as follows: LRC is large rockfish complex, SRC is small rockfish complex, POP is Pacific ocean perch, WDW is widow rockfish, YWT is yellowtail rockfish, and TYH is thornyhead.
[b]n_j is the sample size.

Table 5. Species-composition estimates for the Newport/first quarter 1992 stratum. Landing estimates are in kilograms of fish.

Rockfish species	Landing estimate ($\hat{Y}_.$)	Percent of stratum total landings	CV (%)
Yellowtail	584 122	38	1
Widow	517 813	33	1
Longspine thornyhead	158 805	10	6
Canary	107 319	7	12
Yellowmouth	40 539	3	35
Bocaccio	37 011	2	25
Darkblotched	23 334	2	20
Pacific ocean perch	21 640	1	8
Shortspine thornyhead	15 851	1	61
Yelloweye	10 685	<1	46
Redstripe	9 686	<1	46
Bank	7 279	<1	83
Rougheye	3 668	<1	42
Silvergrey	2 979	<1	52
Splitnose	1 939	<1	49
Sharpchin	1 677	<1	43
Greenstriped	1 374	<1	49
Aurora	340	<1	36
Rosethorn	79	<1	57
Total	1 546 140	100	

would be subsampling procedures within the SRC market category, where the selection of three to four baskets of fish may be warranted in some situations, depending on the strata of interest and sampling objectives. Future design investigations regarding optimum sampling and subsampling fractions, ones that consider sampling costs, would provide information on efficient sample size allocations.

It is important to note that the designs used in this study for the species- and age-composition sampling programs did not utilize random selection protocols for boat trips, market categories, or baskets of fish. That is, port samplers arbitrarily chose the sampling units at each stage of the design based on suggested sampling rates. This design is commonly referred to as quota sampling. This purposive method of selecting samples is used in most commercial fishery monitoring programs and is a difficult problem to circumvent (Tomlinson 1971; A.R. Sen, Department of Mathematics and Statistics, The University of Calgary, Calgary, AB T2N 1N4, personal communication).

It is evident from sampling theory that the results generated from nonprobability sampling have no definable way of being evaluated statistically, because it is not possible to construct a probability distribution for the sample and thus, the variance of the landing estimates cannot be determined. The issue of nonrandom sampling in commercial fisheries is most often avoided by assuming that boats arrive at a port in a random manner and any selection thereof will produce samples that can be treated as random units. The appropriateness of this assumption could be evaluated by comparing easily obtained characteristics of sampled and unsampled boat trips, such as fishing locales, gears used, and types and total weights of market categories

Table 6. Age-composition summaries for groundfish landings in Oregon, January–December 1991. Landing estimates are in number of fish.

Groundfish species	Age	Landing estimate ($\hat{Y}_{.j}$)	Percent of total landings		CV (%)
Yellowtail rockfish (n_j = 35)[a]	5	3 166	<1		82
	6	41 245	3		24
	7	204 751	16		23
	8	194 155	15		18
	9	121 146	9		23
	10	176 841	13		10
	11	106 203	8		9
	12	78 627	6		15
	13	81 901	6		21
	14	77 285	6		14
	15	79 947	6		25
	16	44 328	3		15
	17	38 624	3		23
	18–59[b]	234 – 15 857	≤1	28–104	
	Total	1 313 938	100		
Widow rockfish (n_j = 138)	4	614	<1		74
	5	64 782	2		19
	6	527 588	13		9
	7	759 068	18		8
	8	647 342	15		6
	9	553 047	13		6
	10	787 852	19		5
	11	365 825	9		8
	12	126 240	3		15
	13	124 780	3		13
	14–39[b]	131 – 44 944	≤1	21–106	
	Total	4 200 305	100		
Canary rockfish (n_j = 17)	5	5 417	1		60
	6	15 907	2		35
	7	50 470	5		33
	8	105 234	11		17
	9	136 758	14		17
	10	114 148	12		16
	11	115 578	12		15
	12	104 466	11		11
	13	57 136	6		14
	14	44 672	5		17
	15	43 621	5		23
	16	17 206	2		33
	17	16 164	2		47
	18	19 640	2		29
	19–41[b]	173 – 12 139	≤1	41–108	
	Total	945 389	100		
English sole (n_j = 14)	2	37 561	1		63
	3	119 995	5		36
	4	316 421	12		25
	5	513 598	20		17
	6	851 558	34		13
	7	484 051	19		12
	8	98 367	4		16
	9	51 100	2		22
	10–23[b]	422 – 24 919	≤1	53–108	
	Total	2 532 791	100		

Table 6 (*concluded*).

Groundfish species	Age	Landing estimate ($\hat{Y}_{.j}$)	Percent of total landings	CV (%)
Dover sole (n_j = 90)	4	1 932	<1	103
	5	9 232	<1	85
	6	135 134	1	25
	7	413 712	2	19
	8	1 560 511	8	11
	9	2 054 728	10	11
	10	2 481 534	13	7
	11	2 889 683	15	6
	12	2 371 421	12	7
	13	1 649 190	8	7
	14	1 309 543	7	9
	15	1 109 327	5	12
	16	944 507	5	10
	17	663 245	3	12
	18	413 662	2	12
	19	313 854	2	16
	20–45[b]	635 – 275 363	≤115–104	
	Total	19 851 884	100	

[a] n_j is the sample size.

[b] Range includes various older ages that individually composed ≤1% of the total landings (in number): 20 ages from 18 to 59 for yellowtail rockfish, 23 ages from 14 to 39 for widow rockfish, 21 ages from 19 to 41 for canary rockfish, 8 ages from 10 to 23 for English sole, and 25 ages from 20 to 45 for Dover sole.

landed. The purpose of these comparisons is not to support or falsify the hypothesis that the selection process generates random samples (i.e., samples are not selected randomly), but rather to provide information that can be used to address qualitatively the extent of possible selection biases, and assess whether the boat-trip samples are representative of the population of boat trips and the assumption of random sampling is reasonable.

The variability of the landing data utilized in stock assessments has received sparse attention in fisheries management (Doubleday 1983), most likely because the inclusion of uncertainty terms at the estimation stage results in a more complicated analysis. Because landing estimates are most often based on data from a subset of a population, there necessarily exists discrepancies, or sampling error, between the sample estimates and the population parameters of interest. There is a tendency, many times unjustified, in stock assessment to treat sample estimates as if they were exact values that had been calculated from census data. This is in part due to the lack of information that exists regarding variance measures for landing estimates of individual species, which are not as a general rule derived and made available to a researcher interested in the status of a particular fish stock.

Recognition of the sampling error or uncertainty associated with the landing data is necessary if realistic and scientific inferences are to be used to establish monitoring policies. That is, interval estimation techniques rather than point estimation methods are appropriate for landing estimates that are based on sampling efforts. For example, the CV of 8% associated with the Pacific ocean perch landing estimate of 21 640 kg (Table 5) translates to a 75% confidence interval (CI) approximately equal to 18 178 to 25 102 kg. This conservative CI was obtained using Tchebysheff's theorem, which states that at least 75% of the observations for any probability distribution will be within two standard deviations of their mean (Scheaffer et al. 1990). Note that the confidence level above is much higher (95%) if the sampling distribution of this sample quantity or any other is assumed to follow approximately a normal distribution. The determination of CIs is particularly important when the species being monitored are allocated to the fishers by means of restrictive quotas, as is the case with Pacific ocean perch, which are currently part of a long-term rebuilding plan. There are other statistical techniques for calculating confidence intervals around sample estimates that are appropriate as well, such as bootstrap analysis (Efron 1982), which has been used to bound catch-per-unit-effort estimates for commercial fisheries (Kimura and Balsiger 1985; Stanley 1992). Regardless of the method used to determine the bound on the error of estimation, each constructed confidence interval can be practically interpreted as an interval estimate of the true population parameter.

A logical first step in determining appropriate quota levels (e.g., acceptable biological catch, ABC, or total allowable catch, TAC) is to document the variability associated with landing estimates that are based on sample data. Subsequently, this aspect of uncertainty connected with the quota estimation process should be considered, at the very least in qualitative terms, during the decision-making process. For example, management decisions could

Table 7. Age-composition estimates for yellowtail rockfish landings in Oregon, January–December 1991. Landing estimates are in number of fish. Between ($\hat{V}_{BTW}$ %) and within ($\hat{V}_{WTH}$ %) boat-trip components of variation are expressed as percentages of the estimated total variance of the landing estimates. n_j = 35 boat-trip samples.

Age	Landing estimate ($\hat{Y}_{.j}$)	Percent of market-category total landings	$\hat{V}_{BTW}$ %	$\hat{V}_{WTH}$ %	CV (%)
5	3 166	<1	97	3	82
6	41 245	3	97	3	24
7	204 751	16	>99	<1	23
8	194 155	15	>99	<1	18
9	121 146	9	>99	1	23
10	176 841	13	99	1	10
11	106 203	8	97	3	9
12	78 627	6	98	2	15
13	81 901	6	99	1	21
14	77 285	6	99	1	14
15	79 947	6	>99	<1	25
16	44 328	3	96	4	15
17	38 624	3	98	2	23
18	15 857	1	98	2	28
19	6 274	<1	99	1	54
20	5 317	<1	97	3	50
21	2 158	<1	98	2	100
22	5 520	<1	97	3	48
23	4 661	<1	97	3	80
24	1 380	<1	98	2	85
25	3 883	<1	97	3	64
26	1 257	<1	98	2	91
28	1 254	<1	98	2	87
29	7 844	1	97	3	52
30	1 271	<1	98	2	63
31	1 086	<1	98	2	99
33	234	<1	98	2	102
35	1 088	<1	98	2	104
39	1 088	<1	98	2	104
41	2 191	<1	98	2	100
42	466	<1	98	2	102
43	1 972	<1	98	2	100
59	918	<1	98	2	103
Total	1 313 938	100			

Note: Age-composition samples were collected only from the YWT market categories. This sampling approach was used because nearly all of the yellowtail rockfish (>99% in weight) was landed in its own market category in 1991.

account for sampling variability and provide stock abundance predictions that are more general, such as proposed quotas that are associated with low-, moderate-, or high-risk outcomes versus statistical probability measures. Research oriented towards analytical treatment of sampling variability in stock assessment models is in its incipient stages and will most likely be an area of important consideration in future fishery work. Researchers in stock assessment would be remiss, in the most benign circumstances, to assume that the error associated with the sampling data can be ignored without any detrimental effects to the long-term welfare of fish stocks.

The results from this study provide the first measures of error associated with species- and age-composition estimates of Oregon groundfish landings. Additionally, variance estimates of age composition presented here represent, for the most part, the only measures of dispersion currently available to stock assessment teams interested in Pacific coast fish stocks. Finally, this study documents the broader application of a sampling design developed for a particular fishery (Sen 1986). This final area of significance is a very important issue that concerns multiple fisheries that are managed jointly, but are not subjected to similar sampling approaches. In general, the Pacific coast states (California, Oregon, and Washington) independently develop and conduct sampling programs for groundfish stocks landed at their respective ports. The states provide estimates of species and age compositions to a central management agency, the

Pacific Fishery Management Council. Groundfish stock assessments are coordinated through the council, which relies on federal, state, and academic scientists to carry out research programs. The intricacies of such a management process often result in available data that are difficult to decipher and review critically. This paper provides evidence that it may be possible to standardize, to some degree, the individual monitoring programs used to sample groundfish landed at Pacific coast ports, which would benefit the stock assessment research conducted on this resource, as well as provide other researchers with data bases that can be more readily accessed and interpreted than currently available.

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Appendix

This appendix presents formulae used to calculate landing statistics. All formulae for mean and total landing estimates follow Sen (1986, see section entitled Estimation Based on Categories

as Domains of Study, formulae for second-stage sampling units of fixed size, p. 412–413). The notation I use follows the general style presented in Sen (1984, 1986); however, the two sets of notation are not identical in all respects, particularly subscripts associated with the various estimators. Additionally, to accommodate sampling protocols unique to Oregon and to address assumptions regarding particular variables used in estimation procedures, I have modified slightly and present in a different form the estimated variances suggested in Sen (1986). Finally, I discuss the applicability of selected estimators to Oregon fishery sample data where appropriate.

Notation

Population parameters are denoted by capital letters. Sample estimators are denoted by small letters or by capital letters with a circumflex, depending on the statistic of interest. Total landing estimates and their associated variances are calculated as the sums of the individual stratum estimates.

Within a port/quarter stratum, to estimate (*i*) the weight of a given species in the landings (species-composition sampling program) or (*ii*) the number of fish of a given age, for a species of interest, in the landings (age-composition sampling program), the following set of parameters and statistics are used.

Boat trips (primary units)

N_j = total number of boat trips for market category j

n_j = number of boat trips sampled for market category j

Notes: (1) $j = 1, 2, \ldots, L$ different market categories (post-stratification units) sampled. For the species-composition sampling program, most often $L = 6$, given of course that all six market categories occurred and each N_j was ≥2 within a port/quarter stratum. For species involved in the age-composition sampling program, $L = 1$, see Methods, Age-composition sampling program.

(2) In general, boat trips contained a single landing for each market category j observed, e.g., a typical boat trip may have consisted of one WDW market category, one YWT market category, and one TYH market category.

Weight of fish

W_{ij} = total weight of all fish species in market category j of boat trip i

$W_{\cdot j}$ = total weight of all fish species in market category j across all boat trips

Note: W_{ij} and $W_{\cdot j}$ are treated as known population parameters, i.e., values are obtained through mandatory fish ticket records maintained by individual processing facilities.

w_{ijk} = weight of basket k sampled from market category j of boat trip i

Baskets of fish (secondary units)

M_{ij} = total number of baskets in market category j of boat trip i

$M_{\cdot j}$ = total number of baskets in market category j across all boat trips

m_{ij} = number of baskets sampled from market category j of boat trip i

Landing variables

Y_{ij} = weight of species y landed in market category j of boat trip i (species-composition sampling program) or number of age y fish, of a particular species, landed in market category j of boat trip i (age-composition sampling program)

$Y_{\cdot j}$ = weight of species y landed in market category j across all boat trips (species-composition sampling program) or number of age y fish, of a particular species, landed in market category j across all boat trips (age-composition sampling program)

$Y_{\cdot\cdot}$ = total weight of species y landed across all market categories and all boat trips

Note: For the age-composition sampling program, $L = 1$; thus, the total landing estimate across all market categories, $Y_{\cdot\cdot}$, was not calculated, see Methods, Age-composition sampling program section.

y_{ijk} = weight of species y in basket k sampled from market category j of boat trip i (species-composition sampling program), or number of age y fish, of a particular species, in basket k sampled from market category j of boat trip i (age-composition sampling program)

The following formulae and discussion pertain to analyses of species composition; however, the estimation procedures can be applied similarly to samples of age composition. To calculate estimates of age composition, the measurement variable becomes the number of age y fish, of a particular species, in the landings, rather than the weight of species y in the landings.

The estimated mean weight of species y per basket in market category j of boat trip i is

$$[A1] \quad \bar{y}_{ij\cdot} = \frac{\sum_{k=1}^{m_{ij}} y_{ijk}}{m_{ij}}$$

and the ratio estimator for the mean weight of species y per basket in market category j across all boat trips is

$$[A2] \quad \bar{y}_{\cdot j\cdot} = \frac{\sum_{i=1}^{n_j} M_{ij}\bar{y}_{ij\cdot}}{\sum_{i=1}^{n_j} M_{ij}} \cong \frac{\sum_{i=1}^{n_j} W_{ij}\bar{y}_{ij\cdot}}{\sum_{i=1}^{n_j} W_{ij}}$$

Equation (A2) is a weighted estimator, where samples from boat trips with market-category landings that are large in size are given more weight in estimating $\bar{y}_{\cdot j\cdot}$ than boat trips with relatively small landing sizes. Given that baskets of fixed weight are selected, similar results will be obtained if the total number of baskets, M_{ij}, is used as a weighting variable for the estimator $\bar{y}_{\cdot j\cdot}$; this method is the standard statistical procedure used in two-stage cluster sampling designs (Sukhatme and Sukhatme 1970; Cochran 1977; Scheaffer et al. 1990).

The only practical way of determining M_{ij} is to estimate it as the total weight of market category j divided by the average weight of the baskets taken, i.e., $\hat{M}_{ij} = W_{ij}/\bar{w}_{ij\cdot}$, where $\bar{w}_{ij\cdot}$ is given by Eq. A5. Both empirical approaches in Eq. A2 utilize a ratio estimator, which is biased; however, the bias is negligible when n_j is large. Note that if the landing size of the market categories has no effect on the species compositions themselves, then weighting each sample mean, $\bar{y}_{ij\cdot}$, will have little influence

on the final estimates generated in Eq. A2. One advantage of using a nonweighted estimator is that the estimated variance procedures are simplified. Additionally, if a nonweighted estimator is appropriate for a two-stage sampling design and the number of secondary units of fixed size is equal across the primary units, then straightforward analysis of variance procedures can be utilized to derive needed statistics, such as variance of the mean, and between and within boat-trip components of variation (see sections 7.10 and 7.11 in Sukhatme and Sukhatme (1970) for a general discussion that addresses these procedures).

The estimator for the total landings, in weight, of species y in market category j is

$$[A3] \quad \hat{Y}_{\cdot j} = \bar{y}_{\cdot j} \hat{M}_{\cdot j}$$

where $\hat{M}_{\cdot j}$ is an estimate of the population total number of baskets in market category j across all boat trips ($M_{\cdot j}$) and is calculated as $W_{\cdot j}/\bar{w}_{\cdot j}$. The estimator $\bar{w}_{\cdot j}$ is the mean basket weight for market category j across all sampled boat trips, n_j, and is calculated as

$$[A4] \quad \bar{w}_{\cdot j} = \frac{\sum_{i=1}^{n_j} \bar{w}_{ij}}{n_j}$$

where $\bar{w}_{ij}$ is the estimated mean basket weight for market category j of boat trip i and is calculated as

$$[A5] \quad \bar{w}_{ij} = \frac{\sum_{k=1}^{m_{ij}} w_{ijk}}{m_{ij}}$$

The estimator $\bar{w}_{\cdot j}$ may be treated as a constant if there exists very little variation in the weights of the replicate baskets selected, within similar market categories, across all of the sampled boat trips, i.e., baskets of fixed weight are selected (Sen 1986). Although the weight of the basket samples from this research varied little over the study period, $\bar{w}_{\cdot j}$ was calculated for each type of market category within a stratum. Estimates for $\bar{w}_{\cdot j}$ in each stratum were all very close to the desired basket weights of 11.34 and 22.68 kg, and all estimates were very precise, with SDs ≤0.65 kg. The estimation procedures outlined here are based on secondary units of fixed size; thus, port biologists should strive rigorously to select basket weights that are as similar as possible. Violations regarding the assumption of fixed basket weights will introduce additional components of bias and variability associated with the generated statistics.

The estimator for the total landings, in weight, of species y across all market categories is

$$[A6] \quad \hat{Y}_{\cdot\cdot} = \sum_{j=1}^{L} \hat{Y}_{\cdot j}$$

The estimated variance of $\bar{y}_{\cdot j}$ is

$$[A7] \quad \hat{V}(\bar{y}_{\cdot j}) \cong \left(\frac{N_j - n_j}{N_j}\right)\left(\frac{1}{n_j \hat{\bar{M}}_{\cdot j}^2}\right) s_{1j}^2 + \left(\frac{1}{n_j N_j \hat{\bar{M}}_{\cdot j}^2}\right) \sum_{i=1}^{n_j} \hat{M}_{ij}^2 \left(\frac{\hat{M}_{ij} - m_{ij}}{\hat{M}_{ij}}\right) \frac{s_{2ij}^2}{m_{ij}}$$

where

$$s_{1j}^2 = \sum_{i=1}^{n_j} \hat{M}_{ij}^2 \frac{(\bar{y}_{ij\cdot} - \bar{y}_{\cdot j\cdot})^2}{n_j - 1}$$

$$s_{2ij}^2 = \sum_{k=1}^{m_{ij}} \frac{(y_{ijk} - \bar{y}_{ij\cdot})^2}{m_{ij} - 1}$$

and

$$\hat{\bar{M}}_{\cdot j} = \frac{\sum_{i=1}^{n_j} \hat{M}_{ij}}{n_j}$$

Note that $\hat{\bar{M}}_{\cdot j}$, the estimator for the population mean number of baskets in market category j per boat trip across all boat trips, $\bar{M}_{\cdot j}$, is a poor statistic when (*i*) there is a large amount of variation among the estimates of total number of baskets in market category j per boat trip across the entire population of boat trips (the $\hat{M}_{ij}$), and (*ii*) very few boat trips are sampled (n_j is small); therefore, the variances themselves are subject to additional bias when these situations occur. When it is possible to ascertain population parameters, usually after the fishing season, a more appropriate estimator for the population mean number of baskets in market category j per boat trip across all boat trips would be

$$[A8] \quad \hat{\bar{M}}_{\cdot j} = \left(\frac{W_{\cdot j}}{\bar{w}_{\cdot j}}\right)\left(\frac{1}{N_j}\right) = \frac{\hat{M}_{\cdot j}}{N_j}$$

The estimated variance of $\hat{Y}_{\cdot j}$ is

$$[A9] \quad \hat{V}(\hat{Y}_{\cdot j}) \cong (\hat{M}_{\cdot j})^2 \hat{V}(\bar{y}_{\cdot j})$$

Formulae for $\hat{V}(\hat{Y}_{\cdot j})$ that include expansion variables based on the total number of boat trips, N_j, rather than the estimated total number of baskets in market category j for the entire population of boat trips, $\hat{M}_{\cdot j}$, may replace Eq. A9 when the appropriate information becomes available; these are standard methods that can be found in most general sample survey texts (e.g., Sukhatme and Sukhatme 1970; Cochran 1977; Scheaffer et al. 1990). If boat trips are randomly selected at the first stage of sampling and n_j is large, then both empirical approaches will generate similar results.

The estimated variance of $\hat{Y}_{\cdot\cdot}$ is

$$[A10] \quad \hat{V}(\hat{Y}_{\cdot\cdot}) \cong \sum_{j=1}^{L} \hat{V}(\hat{Y}_{\cdot j}) + 2 \sum_{a} \sum_{<b} \mathrm{Cov}(\hat{Y}_{\cdot a}, \hat{Y}_{\cdot b})$$

Landing estimates for species contained in two or more market categories sampled from the same boat trip may depend upon one another (Sen 1986); therefore, the covariance terms should be added to the summed variances in Eq. A10. If only one market category is sampled per boat trip, then the covariance terms will be zero and the issue of covarying species compositions between market categories within boat trips can be ignored (Sen 1986). The above argument is tenable if boat trips, as well as market categories within boat trips are considered independent random variables; otherwise, further investigations are warranted. Where market categories are likely to be different in their species compositions and weights, as is the case with Oregon landings, a reasonable assumption is to

treat the two or more sampled market categories as independent (Sen 1984; Parker and MacCall 1990). Given that port biologists for Oregon have on occasion sampled more than one market category per boat trip, the issue of possible covariance was examined further. Diagnostic analyses regarding the landing observations indicated that there were no apparent linear associations between the landing estimates for species in different market categories sampled from the same boat trip. The analyses showed no evidence that the covariance term would have a significant influence on the estimated variances derived in Eq. A10. Given the above findings, Eq. A10 simplifies to

$$[A11] \quad \hat{V}(\hat{Y}_{..}) \cong \sum_{j=1}^{L} \hat{V}(\hat{Y}_{.j})$$

The between ($\hat{V}_{BTW}$) and within ($\hat{V}_{WTH}$) boat-trip components of $\hat{V}(\bar{y}_{.j})$ are simply

$$[A12] \quad \hat{V}_{BTW} = \left(\frac{N_j - n_j}{N_j}\right)\left(\frac{1}{n_j \hat{\bar{M}}_{.j}^2}\right) s_{1j}^2$$

and

$$\hat{V}_{WTH} = \left(\frac{1}{n_j N_j \hat{\bar{M}}_{.j}^2}\right) \sum_{i=1}^{n_j} \hat{M}_{ij}^2 \left(\frac{\hat{M}_{ij} - m_{ij}}{\hat{M}_{ij}}\right) \frac{s_{2ij}^2}{m_{ij}}$$

where s_{1j}^2 and s_{2ij}^2 are given by Eq. A7. The between and within boat-trip components of variation are presented in the results as percentages of $\hat{V}(\bar{y}_{.j})$, and noted as $\hat{V}_{BTW}$ % and $\hat{V}_{WTH}$ %, for the between and within components, respectively.

Replacement of herbivore zooplankton species along gradients of ecosystem productivity and fish predation pressure

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Abstract: In general the pooling of major taxa did not provide a basis for classifying zooplankton communities in 342 large Norwegian lakes, as neither cladocerans nor calanoids varied systematically with lake productivity or fish predation pressure. At the species level, most herbivorous cladocerans and calanoids, which constituted three quarters of the metazoan zooplankton biomass, differed in their preference for lake productivity and fish community and could be distinctly grouped according to these variables by canonical correspondance analysis. The analysis pointed out one oligotrophic and one eutrophic specialist among the herbivorous cladocerans, while two of the calanoids were oligotrophic specialists. The biomasses of cladocerans, calanoids, or daphnids were poorly correlated with both lake productivity and fish predation, whereas shifts in average size and species distribution could be attributed to these variables. At low lake productivity, chemical variables such as pH and Ca, as well as the species' physiological adaptations, appear as the main determinants for the competitive advantage and relative success of herbivorous species. Fish community composition changes with increasing lake productivity, but only at very high fish predation intensity (cyprinid communities) did the effects of predation become the main determinant of the zooplankton community, superimposed on the lake productivity.

Résumé : Le regroupement par taxons principaux n'a pas permis, en règle générale, de classifier les communautés de zooplancton de 342 lacs de Norvège puisque ni la productivité du lac ni la pression exercée par les poissons prédateurs ne semble influer de façon systématique sur les cladocères et les calanoïdes. La plupart des espèces de cladocères et de calanoïdes herbivores, qui comptaient pour les trois quarts de la biomasse des métazoaires du zooplancton, se distinguaient cependant l'une de l'autre par la « préférence » manifestée pour une certaine productivité et une certaine communauté ichtyenne, et pouvaient être groupées en fonction de ces critères grâce à l'analyse de corrélation canonique (ACC). L'analyse a permis de distinguer un spécialiste des milieux oligotrophes et un autre des milieux eutrophes parmi les cladocères herbivores, alors que deux des calanoïdes étaient des spécialistes des milieux oligotrophes. Nous avons observé une corrélation faible entre la biomasse des cladocères, des calanoïdes et des daphnies d'une part, et la productivité des lacs et la prédation par les poissons d'autre part, alors que les variations de la taille moyenne et de la distribution des espèces pouvaient être attribuées à ces variables. Lorsque la productivité du lac est faible, les variables chimiques comme le pH et la teneur en Ca ainsi que les adaptations physiologiques des espèces semblent être les principaux facteurs déterminants de l'avantage concurrentiel et du succès relatif des espèces herbivores. La composition des communautés ichtyennes évolue en fonction de la productivité, mais les effets de la prédation ne deviennent le principal facteur déterminant des communautés zooplanctoniques, par-delà l'effet de la productivité du lac, que dans les cas de prédation très intense (p. ex., par les cyprinidés).

[Traduit par la Rédaction]

Introduction

As primary production of an ecosystem increases, the consumers will change with regard to production, biomass, and community composition. The total herbivore biomass response to increased production or changes in food availability may in part be balanced by responses among their predators, while changes in community composition associated with increased production may be reinforced by the predators. In pelagic food webs in freshwater, man-made

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enrichment (eutrophication) has caused major changes in the phytoplankton, zooplankton, and fish communities, influencing overall food web structure and ecosystem stability (see Gulati et al. 1990). The most obvious changes have been seen in the phytoplankton community where a transition from small, edible algae to larger and less edible diatoms and filamentous or toxic cyanobacteria is a frequent result of eutrophication. These eutrophic algae are poor food for most zooplankton species (Watson and Kalff 1981; Lampert 1987), resulting in reduced food chain efficiency with less energy transferred to higher trophic levels.

In general there is a positive relationship between phytoplankton and total zooplankton biomass up to a certain level of system enrichment (McCauley and Kalff 1981; Hanson and Peters 1984). Changes at the community level are less predictable. Often a relative decrease in biomass of calanoid copepods versus cyclopoids and cladocerans are predicted (McNaught 1975; Patalas 1972; Muck and Lampert 1984) along with an increasing proportion of microzooplankton (rotifers and ciliates) (Brooks 1969; Bays and Chrisman 1983). In addition a general tendency toward reduced intraspecific body size as well as shifts toward smaller species is generally predicted (Brooks and Dodson 1965; Brooks 1969; Zaret 1980), mainly attributed to increased fish predation pressure. Dominance of large-bodied species in nutrient-poor waters may be attributed to the competitive superiority of these species at low food concentrations (Gliwicz 1990), and thus, the transition from large to smaller species could be related to abundance, community composition, and nutritional quality of food species as well as to changes in the predator community. On eutrophication, there is frequently a shift in fish species composition. In many Scandinavian and North European waters, salmonids and perch are typically replaced by dense schools of highly specialized zooplanktivorous cyprinids (Faafeng and Nilssen 1981) thus improving the competitive advantage of smaller zooplankton species.

The response of the zooplankton community to ecosystem enrichment is related to a variety of factors including changes in abiotic parameters, food availability, changes in predation, or interspecific competition. The relative importance of these factors is not easily determined. Further, most of these factors are likely interrelated and, to some extent, may also work in the same direction.

Some of the studies on community changes along gradients of primary productivity deal only with major taxa such as cladocerans, cyclopoid and calanoid copepods, and rotifers (Pace 1986). Alternatively, a distinction is made between microzooplankton and macrozooplankton with some arbitrary cut-off between the two categories (often with developmental stages of macrozooplankton included in the microzooplankton group). Recognizing the widespread variability within these categories, such generalizations may say little about the real community differences among lakes of different trophic state or changes within lakes undergoing eutrophication. Some data (Rognerud and Kjellberg 1984; Pinel-Alloul et al. 1990) point toward consistent species changes that may disappear when species are grouped into higher taxonomic categories.

Here we present data from a large number of Norwegian lakes and relate the distribution of major pelagic zooplankton taxa to lake productivity and fish predation pressure. At the species level we focus on the herbivorous calanoids and cladocerans, in particular *Daphnia* spp. We discuss species preferences and replacement along a productivity gradient, trying to assess whether changes in community structure are attributable to productivity changes per se or to fish predation.

Materials and methods

During 1988, 342 lakes in Norway were sampled in May, June, July, and August/September for the following variables: total phosphorus (TP), total nitrogen (TN), and chlorophyll *a* (Chl. *a*). Zooplankton were sampled in July and August/September, as many of the lakes do not have fully developed zooplankton communities before July. For the present analysis we have selected only samples from July and August/September for all variables, to match the zooplankton data. Calcium concentration and pH were determined from samples collected during the autumn survey during thermal circulation.

The lakes represented a range from oligotrophy to eutrophy, with TP in the range 3–246 $\mu g \cdot L^{-1}$, but with the majority of lakes below 50 $\mu g\ TP \cdot L^{-1}$. Most (94%) of the lakes had a surface area >1 km^2 (average 11.8 km^2), and were located at latitudes from 65 to 77°N. The 20 largest Norwegian lakes were included. High-altitude lakes were avoided. All variables were analyzed from integrated samples over the trophogenic zone (two times the Secchi depth; maximum 10–0 m, minimum, 2–0 m). TP and TN were analyzed with a Technicon autoanalyzer after persulfate digestion, and Ca was analyzed by atomic absorption. Chlorophyll *a* was measured spectrophotometrically after acetone extraction.

All species and stages of zooplankton (including rotifers but not unicellular zooplankton) were determined from a 10-L pooled sample from the trophogenic zone (as for the variables above) filtered on a 45-μm net. On a few occasions with highly turbid waters, a smaller volume (5–8 L) was screened. A fixed mean weight was assumed for the various rotifer species as well as for the calanoid and cyclopoid nauplii. For all cladocerans and advanced stages of copepods total length was measured either for each individual, or a representative subsample where the number of individuals of a species exceeded 20. Dry weights (DW) of rotifers and nauplii, and length to DW conversions were derived from Bottrell et al. (1976) and Dumont et al. (1975). Carbon was assumed to constitute 45% of DW (Andersen and Hessen 1991). For all the *Daphnia* spp., data on average body length was based on measurements of the entire sample, or a maximum of 20 individuals.

The fish species composition in all lakes was classified based on either reports, questionnaires, or local catch data. Seven functional community classes were originally identified (Table 1), representing supposedly increasing zooplankton predation pressure from class 1–7. A drawback of this classification is the lack of quantitative fish data, but this could not be overcome for all the lakes investigated. In general, fish density also commonly increases from class 1 to 6, reflecting lake productivity to some extent. In Norwegian lakes, cyprinids are normally restricted to

Table 1. Classification of functional fish community categories, tentatively ranked from low (class 1) to high zooplankton predation pressure (class 6). Class 7 is dominated by invertebrate predators.

Class	Key species	Typical communities	No. of lakes
1	Brown trout (*Salmo trutta*)	No other species present	43
2	Arctic char (*Salvelinus alpinus*) or perch (*Perca fluviatilis*)	Brown trout and arctic char or brown trout and perch	100
3	Whitefish (*Coregonus lavaretus*) or stickleback (*Gasterosteus aculeatus*)	Brown trout, whitefish and perch or brown trout and stickleback	106
4	Smelt (*Osmerus eperlanus*) and cisco (*Coregonus albula*)	Smelt, cisco, brown trout, whitefish	14
5	Whitefish and (or) cisco (shallow, turbid lakes)	Whitefish, cisco	5
6	Various cyprinids	Various composition of the species: roach (*Rutilus rutilis*), bleak (*Alburnus alburnus*), rudd (*Scardinius erythrophtalmus*) or dace (*Leuciscus leuciscus*)	65
7	*Mysis relicta*	Brown trout, arctic char	3

southeastern regions, where the most eutrophic lakes are found. Owing to migration barriers, the more western and northern eutrophic lakes do not have cyprinid populations, however, allowing a statistical separation of these two parameters.

All statistical analyses were done with JMP 2.0 (SAS Institute Inc., Cary, N.C.), except canonical correspondence analysis which was done with an in-house implementation (in MATLAB 4.1) of the algorithm given by Ter Braak and Prentice (1988). To assess distribution at the species level, we chose canonical correspondence analysis (CCA), which is a direct gradient analysis technique introduced by Ter Braak (1986). For a detailed treatment on CCA and its relationship to other ordination techniques, we refer those interested to Ter Braak and Prentice (1988) and references therein; we will only reiterate some highlights of the method here.

While (unconstrained) ordination techniques like principal component analysis (PCA) or detrended correspondence analysis (DCA) are eigenvector extraction techniques using only a matrix of species abundances by sites (the *Y*-matrix) as input, CCA is a constrained ordination technique in the sense that ordination axes extracted from the *Y*-matrix are forced to be multiple linear regressions on a *Z*-matrix of environmental variables by site. Ter Braak (1986) shows that the *Y*-matrix species loadings and *Z*-matrix regression weights from a CCA constitutes a biplot that can be interpreted as a weighted least squares approximation of the weighted averages of the species with respect to the environmental variables. Under certain simplifying assumptions with regards to species packing models, these approximations will furthermore be equivalent to maximum likelihood estimates of unimodal, Gaussian species distributions from Poisson-distributed species abundance data (Ter Braak 1986). Simulation studies indicate that CCA performs well in non-ideal situations where species distributions can be skewed, where there can be quantitative noise in species abundance data, where environmental variables can be highly correlated, and where all factors determining species distributions might not be known (Palmer 1993).

The *Y*-matrix in our analysis consisted of mean biomasses (from the July and August/September samples) of the 14 most common cladoceran and calanoid species. Owing to a high incidence of zero biomasses, we used square-root transformations instead of log transformations to lessen the relative importance of the few lakes with very high biomasses. The choice of transformation of the *Y*-matrix is probably quite unimportant as trial runs with untransformed or binary (presence/absence) data seemed to give similar results. For clarity, further arguments for the choice and interpretation of CCA variables are included in the results.

Results

The investigated lakes spanned roughly two orders of magnitude with respect to water quality variables such as Chl. *a*, TP, TN, and total Ca (Fig. 1). All water quality variables except pH were strongly skewed to the right and, thus, became more symmetrical after log transformation. Trophic state variables Chl. *a*, TP, and TN were closely correlated ($r^2 = 0.46–0.76$ on $\log_{10}$-transformed variables; $n = 342$), while $\log_{10}$ (total Ca) had strong correlation to pH ($r^2 = 0.62$, $n = 342$). On the other hand, trophic state variables were not closely correlated to Ca or pH.

Total zooplankton biomass, as well as major taxonomic groups, all spanned roughly three orders of magnitude in the investigated lakes (Fig. 2). Although distributions of major taxonomic groups are clearly overlapping, median biomasses could be ranked as cladocerans > calanoids > cyclopoids > rotifers, with median cladoceran biomass being one order of magnitude higher than median rotifer biomass. Zooplankton biomass distributions were also strongly skewed to the right, but became quite symmetrical after log transformation (Fig. 2).

The effects of lake productivity and fish predation on zooplankton standing stocks were assessed by analysis of

Fig. 1. Scatterplot matrix of water quality parameters for the investigated lakes (means of July and August/September samples; n = 342). Dashed lines are fitted 95% bivariate normal density ellipses.

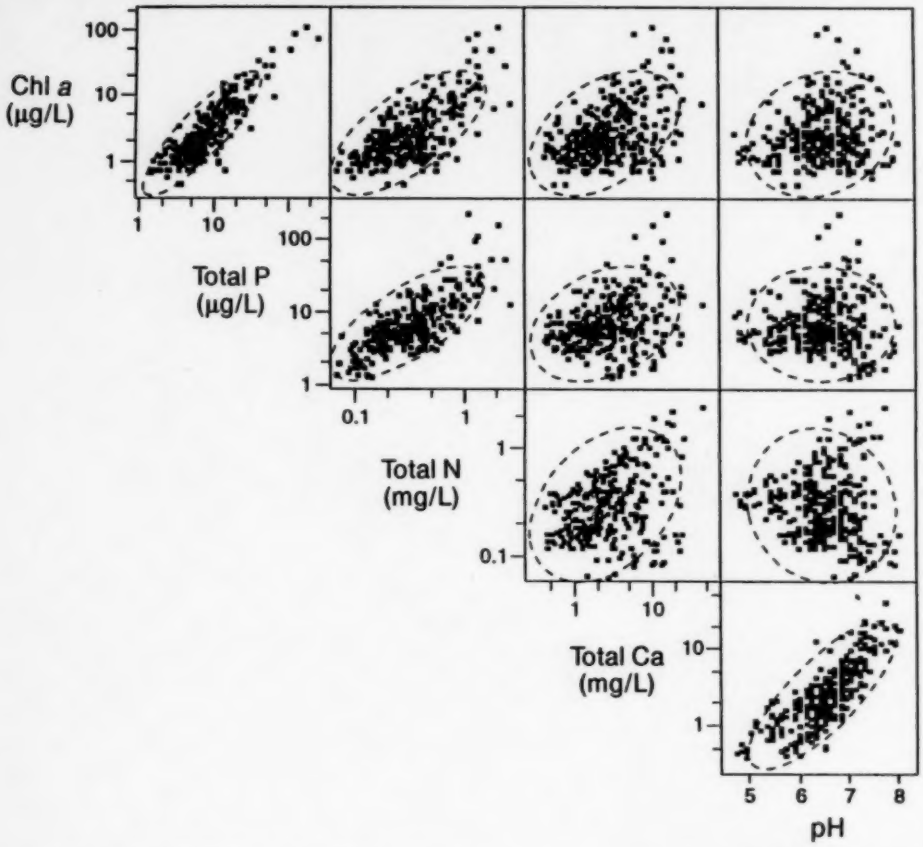
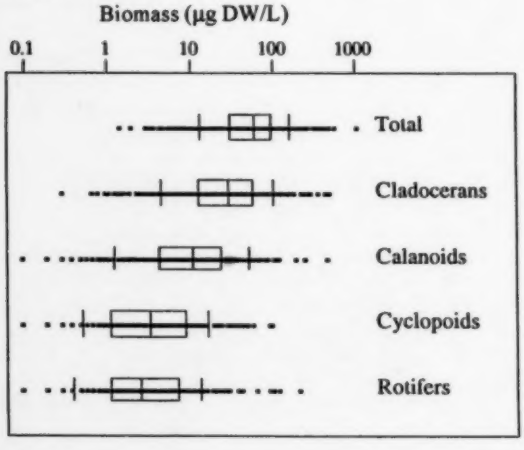


Fig. 2. Box plots of biomass distributions for total zooplankton and major taxonomic groups (means of July and August/September samples). Boxes mark medians and interquartile ranges, while vertical lines mark the 10 and 90% percentiles. Box heights are proportional to sample sizes.



covariance (ANCOVA) using TP and fish community class as independent variables. The choice of TP as lake productivity index is somewhat arbitrary; because TP is closely correlated with Chl. *a* and TN (Fig. 1), we could in fact have used any of these variables. Both total P concentrations and zooplankton biomasses were log transformed to reduce skewness. The three fish community classes with fewest members (classes 4, 5, and 7; totalling 22 lakes) were excluded from this analysis to make the data set more balanced with respect to fish communities.

Total P had a highly significant effect on zooplankton biomass for all major taxonomic groups (Table 2). Regression slopes were homogeneous with respect to fish community class (i.e., no significant fish × TP interaction terms), and fairly similar (ranging from 0.41 ± 0.12 (mean ± SE) for cyclopoids to 0.71 ± 0.12 for rotifers; Table 2). Mean zooplankton biomass was thus roughly proportional to the square root of TP (slope (b_{TP}) ~ 0.5; Table 2), which means that zooplankton biomass is expected to vary by only a factor of 10 when TP varies by a factor of 100, as in the investigated lakes. In other words the major part of the observed three-decade variability in zooplankton biomass (Fig. 2) is left unexplained by TP and fish community class (explained proportion of variance (r^2; coefficient of

Table 2. Results of ANCOVAs with $\log_{10}$ (zooplankton biomass) as dependent variable and $\log_{10}$ (total P) (TP) and fish community class (fish) as independent variables.

Dependent variable	r^{2a}	n	$b_{TP}{}^{b}$	$F_{TP}{}^{c}$	p^{d}	$F_{fish}{}^{e}$	p^{d}
Total zooplankton	0.179	309	0.59±0.08	55.06	<0.0001	0.73	0.5344
Cladocerans	0.071	309	0.47±0.10	20.67	<0.0001	0.93	0.4256
Calanoids	0.088	257	0.58±0.13	19.64	<0.0001	0.14	0.9363
Cyclopoids	0.147	268	0.41±0.12	11.31	0.0009	4.37	0.0051
Rotifers	0.168	243	0.71±0.12	35.18	<0.0001	3.16	0.0255

Note: Fish community classes 4, 5, and 7 are excluded owing to small sample size. Sample size variation is due to absence of certain major taxonomic groups in some lakes.

[a] r^2, whole-model coefficient of determination.

[b] b_{TP}, common TP regression slope (±SE).

[c] F_{TP}, TP effect F-ratio (1, n − 1 df).

[d] Probability of F = 0.

[e] F_{fish}, fish effect F-ratio (3, n − 3 df).

determination; Table 2) ranged from 18% for total zooplankton down to only 7% for total cladocerans).

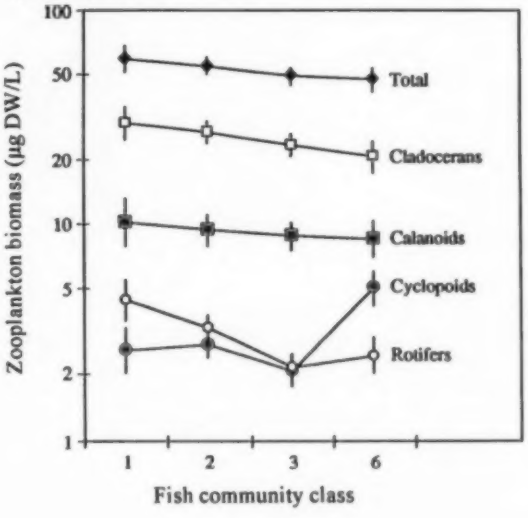
Least-square means (Fig. 3) reflect average zooplankton biomasses corrected for the effects of TP as an index of lake productivity. Cladocera generally made up >50% of zooplankton biomass, while calanoid copepods constituted ~25% and cyclopoid copepods and rotifers 5–15% each. Fish community effects on biomasses of major taxonomic units were only significant for total rotifers and total cyclopoids (Table 2). The apparently inverse relationship between rotifers and cyclopoids with increasing fish predation pressure (Fig. 3) could either reflect a prey–predator relationship or a competitive interaction between rotifers and naupliar stages of cyclopoids. A remarkably low proportion of cladocerans, and increased relative success of calanoids was recorded in the few localities dominated by *Mysis*.

The two major groups of herbivores, cladocerans and calanoid copepods, constituted together, on average, close to 75% of metazoan zooplankton biomass, and were represented in nearly all lakes. Consequently, we tried to investigate in closer detail the distribution pattern of individual species in these two quantitatively important groups.

For this purpose we chose canonical correspondence analysis (CCA). As in the ANCOVAs above, we excluded all lakes belonging to the three smallest fish community classes such that membership in any of the remaining classes could be coded as four binary variables. When the first of these dummy variables (coding for membership in fish community class 1) is deleted, the remaining three columns constitute a proper design matrix that can be used to express effects of fish community class (relative to class 1). The Z-matrix in our analysis, thus, consisted of the three columns of the design matrix for fish community class and the five columns of water quality parameters shown in Fig. 1. Following Palmer (1993), we log transformed the data for TP, TN, Chl. *a*, and Ca, while pH data were left untransformed.

Ter Braak and Prentice (1988) state that nonlinear methods like CCA are more appropriate than linear methods like PCA when gradients are greater than ~3 SD units. The site scores for the first two CCA axes in our analysis spanned 5.1 and 5.9 SD, respectively. The two first ordination axes had

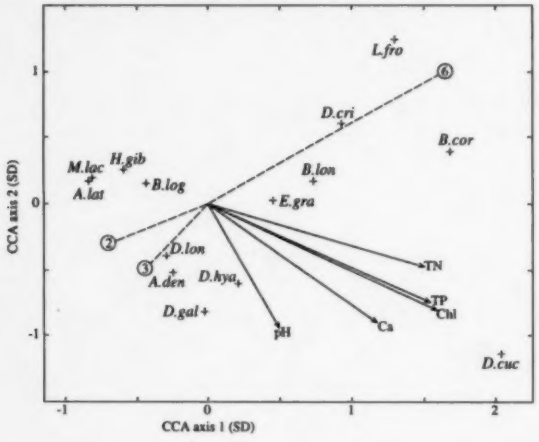
Fig. 3. Least-square means of fish effects in ANCOVA of $\log_{10}$ (zooplankton) on $\log_{10}$ (total P) and fish community class (cf. Table 2). Error bars are ±1SD of the least-square means.



eigenvalues of 0.43 and 0.20, which was equivalent to 79% of the total variance that could be explained by the eight variables of our Z-matrix. The first ordination axis of the species abundance variation that remained after the effects of the environmental variables had been taken out (the first residual axis), had an eigenvalue of 0.40. In other words, at least half of the variation in species abundance was left unexplained by the environmental variables used in the CCA ordination, which seems to be comparable with what is considered typical for terrestrial community data (Ter Braak and Prentice 1988).

The biplot from the CCA (Fig. 4) shows that the environmental variables form two distinct gradients that can be tentatively interpreted as water productivity (along the direction of the TP and Chl. *a* vectors) and fish community structure or predation pressure (along the direction from

Fig. 4. Biplot of species loadings and regression weights from canonical correspondence analysis of square-root transformed zooplankton biomass data. Continuous environmental variables (Ca, Chl. *a*, pH, TN, TP) marked by directed arrows. Categorial environmental variables (fish community classes 2, 3, and 6) marked by numbers inside circles and dashed lines. Species scores marked by crosses and species name abbreviations are A. den, *Acanthodiaptomus denticornis*; A. lat, *Arctodiaptomus laticeps*; B. cor, *Bosmina coregoni*; B. lon, *B. longirostris*; B. log, *B. longispina*; D. cri, *Daphnia cristata*; D. cuc, *D. cucullata*; D. gal (*D. galeata*), D. hya, *D. hyalina*; D. lon, *D. longispina*; E. gra, *Eudiaptomus gracilis*; H. gib, *Holopedium gibberum*; L. fro, *Limnosida frondosa*; M. lac, *Mixodiaptomus laciniatus*.



fish community classes 2 and 3 to class 6). Within these two gradients, we can identify three or four groups of zooplankton species. At the lower end of the productivity gradient, in lakes without cyprinid dominance, we have a group of two calanoid and two cladoceran species that seem to be characteristic for oligotrophic lakes: *Mixodiaptomus laciniatus*, *Arctodiaptomus laticeps*, *Holopedium gibberum*, and *Bosmina longispina*. In more productive noncyprinid lakes we have a group consisting of the three largest *Daphnia* species (*D. galeata*, *D. hyalina*, and *D. longispina*) and the largest calanoid (*Acanthodiaptomus denticornis*), which might be inferred to be quite predation sensitive. At the other end of the fish predation gradient we find a group of small-bodied species that appear to be comparatively predation resistant (*Bosmina coregoni*, *Daphnia cristata*, *Limnosida frondosa*, and possibly also *Bosmina longirostris* and *Eudiaptomus gracilis*). The last species in our analysis, *Daphnia cucullata*, stands out in an isolated position at the upper end of the productivity gradient.

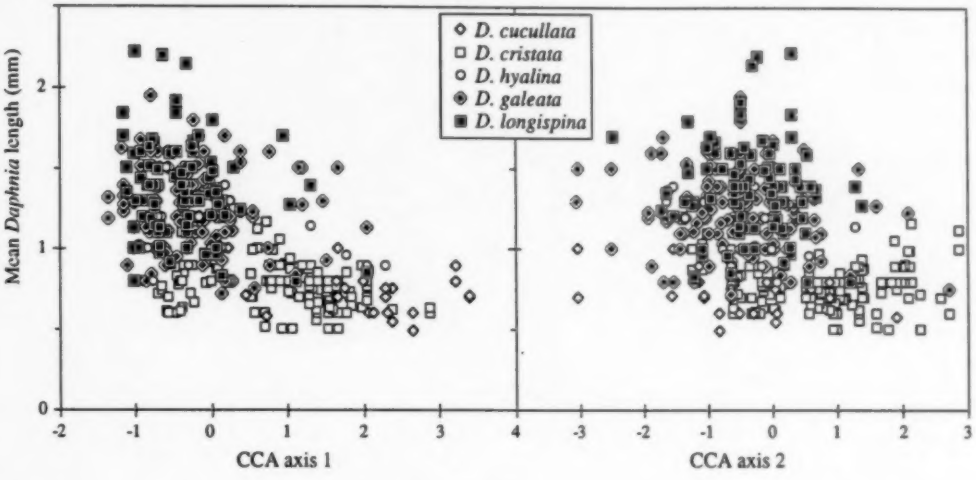
The interpretation of CCA ordination axis 1 as a fish predation pressure gradient is evident from Fig. 5; there is a clear tendency for decreasing *Daphnia* body size (and thus decreasing vulnerability to visual predators) along the first ordination axis, while there is no such pattern along the second ordination axis. Although much of the twofold variation (from >2 mm down to ~1 mm) in maximal body length at a given CCA axis 1 site score is due to species replacements (large towards small species), there are also some interesting patterns in intraspecific body-size plasticity. *Daphnia cucullata*, and especially *D. cristata*, appear to have more or less the same mean body size over the whole gradient, and remain small even at low fish predation pressure. *Daphnia galeata*, *D. hyalina*, and *D. longispina* show more pronounced body-size variability with a clear tendency for reduced body length with increasing fish predation pressure. *Daphnia galeata* seems to be the most flexible of these species, and does in fact persist in several lakes with high fish predation pressure.

Discussion

With the precaution that the surveyed lakes are biased towards nutrient-poor localities and that seasonal succession is not accounted for, there seems to be no basis for a general classification of major taxa as being either oligotrophic or eutrophic according to their preference for localities. Neither the cladocera nor the calanoids or rotifers had any clear preference for nutrient-poor or nutrient-rich lakes. The proportion of cyclopoids in the total zooplankton increased in the most eutrophic lakes, but this was attributed to fish predation rather than lake productivity. Still, it confirms the general expectation of increased relative success of this group in eutrophic lakes (Brooks 1969; McNaught 1975; Langeland and Reinertsen 1982). A number of studies have also suggested a general transition from calanoids to cyclopoids with increasing lake productivity (Gliwicz 1969; Patalas 1972; Bays and Crisman 1983; Rognerud and Kjellberg 1984), and this has been related to changes in food size spectra (Pace 1986). Some studies also report increased relative success of cladocerans with eutrophication (Geller and Müller 1984; Rognerud and Kjellberg 1984), and it is a frequent assumption that microfiltrators may have increased success in more productive lakes. This is attributed to the size spectrum of the food particles, where species capable of retaining smaller particles (microalgae and bacteria) would be favoured when the share of edible, medium-sized algae is reduced or replaced by less edible species (Gliwicz 1977; Hessen et al. 1986). Geller and Müller (1981) proposed this as a unifying explanation for a supposedly increasing success of *Daphnia* spp. in more eutrophic waters. Although this might seem a reasonable a priori assumption, we found no such general division in micro- and macro-filtrator communities as related to lake productivity. A contrasting response would be the inhibition of nonselective filter feeders like *Daphnia* when the share of filamentous or toxic blue-green algae increase (cf. Gliwicz 1977), but we did not observe any significant relationship between daphnids (or other main groups) and the share of blue-green algae. In general, our data do not support any generalization relating changes in trophy to success of the major zooplankton taxa.

An examination of species-level responses of the two major herbivore groups unravels some of the contrasting effects when considering pooled groups of species. First, both groups include both oligotrophic and eutrophic species, and second, most species occur over a wide range of lake productivity and should be considered generalists for the present range of lake productivity. This also seems to hold

Fig. 5. Mean lengths of five *Daphnia* species in relation to site scores on the two first ordination axes of the canonical correspondence analysis shown in Fig. 4.



for copepods and rotifers, which comprise a few specialists but mostly generalists (Pejler 1965; Bérzins and Peijler 1989). Thus, depending on species composition in surveyed lakes, contrasting responses may be expected when classifying major taxa with pooled species.

Although most species occur over a fairly broad range of lake productivity there is a transition in dominant species when going from low to high productivity. The eutrophic specialist among the daphnids was *D. cucullata*. In addition to predation resistance due to small body size, the direct association of this species with eutrophic lakes might also be related to proposed adaptions in this species for avoiding inedible net phytoplankton (Gliwicz 1980). Several authors have suggested increased relative success of species capable of filtering bacteria during eutrophication. Geller and Müller (1981) proposed this as a main reason for the success of *D. cucullata* in eutrophic lakes. This may not be a unifying explanation as species like *D. longispina* and *Diaphanosoma brachyurum*, which are highly efficient bacterivores (Geller and Müller 1981; Hessen 1985; Hessen et al. 1990), show no preference for eutrophic lakes and frequently occur in oligotrophic lakes. Also the calanoid *Eudiaptomus gracilis* and bosminids like *B. longirostris* and *Bosmina coregoni*, which are not considered as efficient bacterivores (DeMott 1985; Hessen 1985; Hessen et al. 1990), are common inhabitants of eutrophic lakes. Macrofiltrators like *Holopedium*, *Eudiaptomus*, and *Bosmina* are also frequently dominant species in humic lakes with a high bacteria:phytoplankton ratio (Hessen et al. 1990). In contrast, the assumed success of cyclopoids in eutrophic lakes has been attributed their ability to select suitable food in an environment with a high frequency of low-quality food (Pace 1986), but note that cyclopoids may show contrasting responses on eutrophication (Pejler 1965; Hessen et al. 1986), and a common species like *Cyclops scutifer* is considered an oligotrophic indicator (Pejler 1965).

The competition component may not be easily extracted. With few exceptions, the data do not indicate that herbivores assumed to share the same food sector spectrum in general avoid cooccurrence. This may be more pronounced at low food abundance. While most Cladocera have food thresholds below which respiratory costs exceed net intake of carbon (Muck and Lampert 1984; Bohrer and Lampert 1988), the calanoids may sustain their density at very low food levels owing to low respiratory costs. It is similarily demonstrated that *Bosmina* do better at low food concentrations than do *Daphnia* (Urabe and Watanabe 1990). Although this may result in increased success of *Bosmina* and calanoids at the expense of *Daphnia* during periods of low food, it does not result in a general divergence with bosminids and calanoids in nutrient-poor, and *Daphnia* in nutrient-rich lakes. There are, however, contrasting reports on the effects of food quantity. While there is experimental and theoretical evidence for increased relative success of small-bodied species at the expense of larger species as the nutrient status of the environment declines (Neill 1975; Lynch 1977, 1978), the classical size-selective hypothesis (Brooks and Dodson 1965; Vanni 1986) suggests the reverse. In support of this hypothesis, Gliwicz (1990) demonstrated a close correlation between body size and threshold food concentration in various daphnids. This could offer a complementary explanation to the increasing success of large species at low nutrient levels, but in nature such patterns are obscured by the complex sources of nutrition as well as the occurrence of vertebrate and invertebrate predators.

Straight community responses to eutrophication are also obscured by the fact that food quality in terms of phytoplankton biochemistry or stoichiometry may be superimposed on food quantity (Sterner and Hessen 1994). Non-selective grazers and species with high specific requirements for some mineral or biochemical components would obviously be disfavoured by a high proportion of nutrient deficient algae. If these factors temporarily are superimposed on effects caused by food quantity or algal community composition, it would counteract clear-cut patterns in lake productivity preference.

Eutrophication is further accompanied by a variety of physicochemical factors that could influence the relative success of species. pH has previously been suggested as a strong species-specific regulating parameter, but only on the extremes of the scale (>9.5, Potts and Fryer 1979; Hessen and Nilssen 1983; <5.5, Hobæk and Raddum 1980; Pinel-Alloul et al. 1989). Although few lakes in the present study belong to these extreme categories, pH nevertheless appeared to impact zooplankton community composition, in line with the data of Yan et al. (1988). The pH effect is not easily separated from the Ca effect (Tessier and Horwitz 1990), and the apparently positive contribution from Ca concentrations on several species should call for some attention. It is likely that freshwater decapods with a heavily calcified exoskeleton may suffer a direct Ca limitation (cf. Hessen et al. 1991), and this could hold also for crustacean zooplankton in Ca-poor lakes (median Ca: 2.4 $mg \cdot L^{-1}$). Tessier and Horwitz (1990) observed a transition from large crustaceans to small rotifers with decreasing water hardness, and related this to Ca deficiency in the crustaceans. Various species have a variable Ca content, ranging from near 3 $mg \cdot g^{-1}$ DW in *Daphnia* to 0.06 in copepods (Yan and Mackie 1989*a*, 1989*b*). Most of the Ca is probably associated with the carapace (Yan and Mackie 1989*a*), and thus at least some proportion of this body Ca must be regenerated after each molt. It is not unreasonable that some of the heavily calcified cladocerans may suffer from Ca deficiency at low ambient Ca concentrations, as the major source of Ca probably is from water and not food (Cowgill et al. 1986). As for fish predation, it is not straightforward to separate the effects of Ca content and lake productivity as these follow closely, and the species' ranking for Ca preference is principally in accordance with that for productivity preference. A test on the distribution of *D. cucullata* demonstrated, however, that there are a number of eutrophic localities where this species would be expected to occur (>10 μg Chl. $a \cdot L^{-1}$), but where Ca concentrations apparently were too low (<4 $mg \cdot L^{-1}$) to support growth of this species. This is in line with the statistical analysis, and pinpoints Ca as a possible limiting element in softwater lakes.

The tentative fish predation pressure, expressed as fish community classes, had different effects on the different taxa, but major effects were only seen at the highest predation pressure. The share of *Daphnia* spp., normally regarded as highly susceptible to fish predation, was not correlated with tentative fish predation. There were, however, pronounced effects on body size accompanying shifts at the species level. It is reasonable to expect some inherited life-strategy components to play a role in the relative success of large or small body sizes at various food levels, thus determining competitive outcome at low predation levels. Thus, although succession of herbivores certainly may be determined by their different abilities to consume different algal species (Irvine 1986; Gulati 1990), the present study indicates that inter- and intra-specific body size, and hence, *Daphnia* species succession was related to fish predation pressure rather than lake productivity per se (cf. Fig. 5).

The various species' ability to withstand fish predation hinge to a great extent on their body-size plasticity. For example, *D. longispina* with the least variability in average body size would be more susceptible than *D. galeata*, although they are equally large at low fish predation. Thus, the reason why *D. longispina* apparently is the most oligotrophic of the surveyed *Daphnia* species could largely be ascribed to the coupling between lake productivity and fish predation pressure, as this species may be successful even at highly eutrophic conditions when released from fish predation (Hessen et al. 1986). More specifically, the minimum reproductive size for a given species will determine its ability to withstand size-selective predation. Thus again, species with a pronounced plasticity regarding minimum reproductive size like *D. galeata* will be the survivors as predation pressure increases.

In conclusion, within all major taxa there are different species preferring both high and low lake productivity. Thus within the range of lakes included in this survey, there seems to be little foundation for classifying lakes according to their share of cladocerans or calanoids. With the exception of the possible physiological constraints of a few species, it appears that most herbivore species seem able to exist over a wide range of lake productivity; thus, the apparent preference of the various species is largely governed by interactions with their competitors and predators. While ecophysiological properties of different species may determine competitive success and, thus, community composition in the oligotrophic range of the spectrum (cf. Gliwicz 1990), fish predation becomes increasingly important to species composition in meso- to eu-trophic lakes. Only in the most eutrophic lakes, dominated by dense population of cyprinids, is fish predation a key determinant of zooplankton community structure. In these lakes, fish predation not only selects for cyclopoids, but also for smaller species and smaller individuals of species.

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Biological and physical variables associated with aggregations of the giant scallop *Placopecten magellanicus*

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Abstract: To identify factors that may determine where aggregations (beds) of giant scallops (*Placopecten magellanicus*) develop, we compared physical and biological conditions in two scallop beds in Port Daniel Bay, Baie des Chaleurs, Gulf of St. Lawrence, with conditions in seven adjacent areas. Gravel substratum predominantly characterized scallop beds. Physical hydrographic conditions and the intensity of asteroid predation were similar within scallop beds and in surrounding areas with few scallops. High *P. magellanicus* spat settlement density was not consistently correlated with existing scallop aggregations, but filamentous organisms, on which scallops preferentially settle, were more abundant in scallop beds, possibly enhancing recruitment. Scallop growth rate varied among areas and was intermediate within scallop beds. Winter flounder (*Pseudopleuronectes americanus*) and Atlantic wolffish (*Anarhichas lupus*) preyed on *P. magellanicus* and may influence scallop distribution. Scallop tethering experiments indicated that risk of predation was low within scallop beds compared with adjacent areas. Scallop mortality was positively correlated with mean density of both rock crab (*Cancer irroratus*) and American lobster (*Homarus americanus*). We conclude that gravel substratum, low decapod predation, and presence of filamentous flora and fauna are critical factors determining scallop aggregation location.

Résumé : Pour déterminer quels sont les facteurs qui peuvent déterminer l'emplacement des gisements de pétoncle géant (*Placopecten magellanicus*), nous avons comparé les conditions physiques et biologiques caractéristiques de deux gisements de pétoncles dans la baie de Port-Daniel (baie des Chaleurs, golfe du Saint-Laurent) à celles qui existent dans sept zones adjacentes. Le substrat de gravier est une caractéristique prédominante des gisements de pétoncles. L'hydrographie et l'intensité de la prédaction par les astéries ne sont pas différentes sur les gisements des conditions qui existent dans les zones avoisinantes où il y a peu de pétoncles. Il n'existe pas de corrélation nette entre la haute densité d'installation du naissain de *P. magellanicus* et les gisements existants, mais les organismes filamenteux, sur lesquels les pétoncles se fixent de préférence, étaient plus abondants sur les gisements et favorisaient peut-être le recrutement. Le taux de croissance des pétoncles variait d'une zone à l'autre et affichait des valeurs intermédiaires sur les gisements. La plie rouge (*Pseudopleuronectes americanus*) et le loup atlantique (*Anarhichas lupus*) se nourrissent de pétoncles et influent peut-être sur leur répartition. Des expériences de fixation de pétoncles ont montré que les risques de prédation sont faibles sur les gisements, comparativement à ceux qui existent dans les régions adjacentes. Nous avons observé une corrélation positive entre la mortalité des pétoncles et la densité moyenne des populations de crabe commun (*Cancer irroratus*) et de homard (*Homarus americanus*). Nous avons conclu qu'un substrat de gravier, une faible prédation par les décapodes et la présence d'une flore et d'une faune filamenteuses sont des facteurs qui déterminent l'emplacement des gisements de pétoncles.

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Introduction

The spatial distribution pattern of a species provides important insight into its population dynamics (Paine 1977). Regular and random patterns are rare, and most species have contagious distributions (Taylor et al. 1978). Factors influencing distribution, which may include physical barriers, predation, competition, and intrinsic behavioral responses, are poorly understood for most species. However, determining the underlying factors controlling spatial distribution patterns at various scales is essential to understanding both the ecology of species and community structure (Menge and Olson 1990).

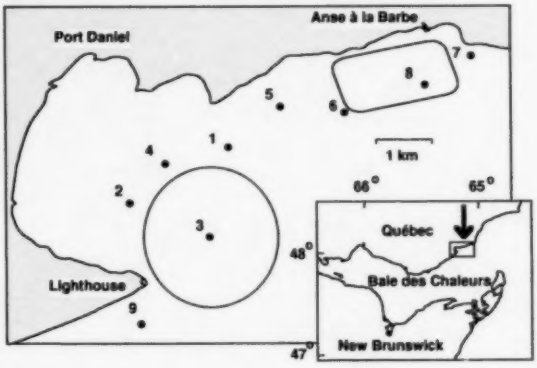
The giant scallop *Placopecten magellanicus* occurs in aggregations, termed beds, on the scale of kilometers throughout its geographic range from North Carolina to Newfoundland (Posgay 1957; Brand 1991). Considerable debate has developed about processes influencing the creation and maintenance of beds of *P. magellanicus* and other scallops. *Placopecten magellanicus* beds, in general, are strongly associated with gravel or gravel and sand substrata (Langton and Robinson 1990; Thouzeau et al. 1991*a*; Stokesbury and Himmelman 1993). On a large geographic scale, scallop aggregations occur in areas where oceanographic conditions facilitate larval retention (Sinclair et al. 1985). On a smaller spatial scale, intensity of spat settlement is not significantly correlated with *P. magellanicus* aggregations (Robinson et al. 1992). Adult concentrations may be sustained by postsettlement migration (Dadswell and Weihs 1990; Robinson et al. 1992). However, studies involving releases of large numbers of tagged *P. magellanicus* indicate that migrations do not occur on the scale of kilometers. Dispersion is mostly in the direction of prevalent currents (Posgay 1981; Melvin et al. 1985). Food quantity and quality, sediment type, and water depth, temperature, turbulence, and current are all considered to be limiting factors for *P. magellanicus* (MacDonald and Thompson 1985*a*, 1985*b*, 1986*a*, 1986*b*; Shumway et al. 1987; Wildish et al. 1987; Thouzeau et al. 1991*b*; MacDonald and Bajdik 1992; Wildish and Saulnier 1992).

Scallop distribution may also be influenced by predation. Cerianthid anemones feeding on *P. magellanicus* larvae may determine adult distribution (Langton and Robinson 1990). Further, predation by crabs (*Cancer irroratus* and *Cancer pagurus*) and sea stars (*Asterias* spp.) strongly influenced the densities and subsequent spatial distributions of bottom-seeded juvenile scallops (Barbeau et al. 1992; Wilson and Brand 1994). Predation rate decreases with scallop shell height, and after reviewing the literature on pectinids, Brand (1991) concluded that predation on adult scallops does not determine aggregation distributions.

The previous studies suggest that the spatial distribution of scallops, on the scale of kilometers, is controlled by a combination of physical and both inter- and intra-specific biological variables, rather than one specific factor. Further, the influence of any one variable within this combination may vary on both spatial and temporal scales.

We investigated factors, over a 2-year period, which may collectively explain why *P. magellanicus* occur in localized aggregations (beds) on the scale of kilometers. We considered both physical variables, such as substratum type, temperature, salinity, current velocity, and direction, and biological variables, such as spat availability, abundance of filamentous biota, food availability, and predation pressure. To evaluate factors that seemed likely to determine where scallops aggregate, we compared characteristics of stations within beds with those of stations in adjacent areas where scallops were scarce.

Fig. 1. Locations of the nine stations in Port Daniel Bay, Baie des Chaleurs, where physical and biological variables were measured during 1991 and 1992. The outlined areas show the approximate boundaries of the scallop beds near the Lighthouse and Anse à la Barbe.



Materials and methods

Our study was preformed in Port Daniel Bay located on the Quebec coast of the Baie des Chaleurs, Gulf of St. Lawrence (Fig. 1). In this region *P. magellanicus* is contagiously distributed (Stokesbury and Himmelman 1993). A scallop bed was defined as an aggregation with a mean density >0.30 scallops·m^{-2}. Two unharvested beds are present, the Lighthouse bed, with a density of 0.57 ± 0.41 scallops·m^{-2} (mean ± SD) and covering an area of ≈5.0 km^2 and the Anse à la Barbe bed, with a density of 0.34 ± 0.31 ind·m^{-2} and covering an area of ≈2.3 km^2 (Stokesbury and Himmelman 1993) (Fig. 1). The Iceland scallop, *Chlamys islandica* is also found in the Baie des Chaleurs but was only present in low numbers (0.03 individuals·m^{-2}) at our study sites. Exploratory dives revealed that it was more abundant below 30 m depth.

We quantified conditions potentially affecting scallop distribution at nine stations in Port Daniel Bay. The stations were selected based on a previous survey in which 50-m transects were used to quantify bottom substrata and the abundance and distribution of scallops (Stokesbury and Himmelman 1993). To eliminate sampling bias, we did not investigate the substratum prior to dropping the station anchor to the bottom (Hurlbert 1984).

At each station we placed an anchor and then estimated scallop densities and substratum types along a 20-m transect heading north from the anchor. The substratum along each transect was classified using five categories of particle size: mud/sand (<1 mm), gravel (0.1–10 cm), cobbles (10–100 cm), boulders (>100 cm), and bedrock. Stations were then characterized according to both the percent

Table 1. Depth, substratum type, and density of *Placopecten magellanicus* at the nine experimental stations in Port Daniel Bay, Baie des Chaleurs. Stations 3 and 8 are within the two scallop beds.

Characteristics	Station								
	1	2	3	4	5	6	7	8	9
Depth (m)	20	16	23	18	19	21	18	22	20
Current ($cm \cdot s^{-1}$)*	7.2 (4.8)	10.2 (6.8)	7.8 (6.0)					6.3 (3.8)	
Current direction (degrees)*	192 (72)	170 (80)	177 (76)					196 (81)	
Scallops·m^{-2}	0	0	0.66	0.05	0.17	0.08	0	0.32	0.19
Scallop size range (mm)			18–138	14–138	40–148	40–110		28–148	15–150
Substratum (% cover)									
Mud	0	0	0	0	0	2	0	0	6
Sand	0	100	2	5	2	0	94	6	3
Gravel	45	0	88	36	67	37	0	81	86
Cobble	13	0	10	59	31	42	0	11	5
Boulders	0	0	0	0	0	17	6	2	0
Bedrock	42	0	0	0	0	2	0	0	0
Predominant substratum	Bedrock	Sand	Gravel	Cobble	Cobble	Cobble	Sand	Gravel	Gravel

*Values are means with standard deviations given in parentheses.

substratum type observed along the transect and the overall dominant substratum type in the surrounding area (Table 1). Scallop density was determined along each transect by sampling a 50-m^2 area (10 × 5 m) for stations 3 and 8, which were inside the Lighthouse and Anse à la Barbe beds, respectively, and a 200-m^2 area (20 × 10 m) for the seven stations outside of the beds (Fig. 1, Table 1).

Current velocity and direction, water temperature, and salinity were recorded at stations 1, 2, 3, and 8 in 1992 using Aanderaa RCM-4S current meters. Temperatures were also recorded during every observational dive at each station in 1991 and 1992.

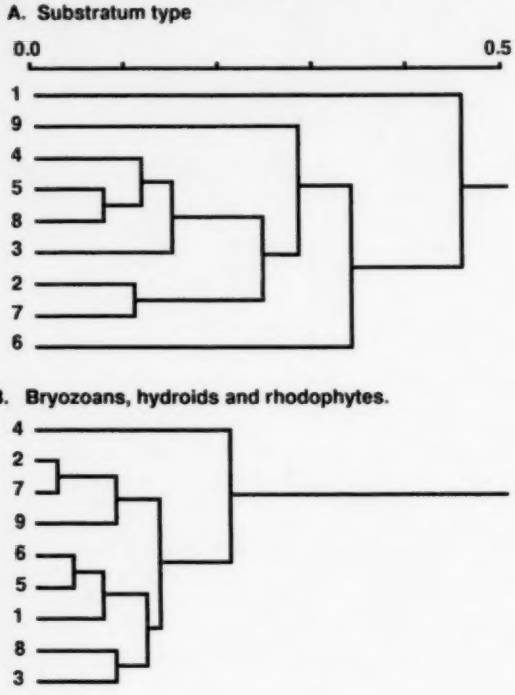
To compare spat recruitment inside and outside the scallop beds, we quantified the number of spat settling on commercial collectors (200 g of monofilament gill netting inside a 2-mm mesh onion bag; Dadswell et al. 1988) at stations 1, 2, and 3 in 1991 and stations 2, 3, 5, 6, 7, and 8 in 1992. At each station, we suspended six collectors 0.5–1.5 m above the substratum. Spat collectors were deployed within 1 week of spawning, determined by weekly examination of scallop gonadal development. Spawning was late in 1991 and began during the second week of September so the collectors were deployed on 13 September and retrieved 10 months later in July 1992. In 1992 spawning began in mid-August. Spat collectors were deployed on 17 August and retrieved 2.5 months later on 29 October 1992. After retrieving the collectors, spat were detached, sieved sequentially through 100-, 1.0-, and 0.25-mm mesh screens, fixed in 5% formalin, and then transferred to 25% ethanol. The 1991 spat (≈10 months old) were readily identified, using a magnifying glass, as *P. magellanicus* or *C. islandica* and counted. The 1992 *P. magellanicus* and *C. islandica* spat (≈2 months old) were smaller and more numerous. We first counted all scallops measuring >1-mm shell height and then estimated the numbers of smaller individuals from a 25% subsample obtained using a Folsom plankton splitter (Van Guelpen et al. 1982). We identified the smaller scallops using a compound microscope and these identifications were verified by a scientist at the Department of Fisheries and Oceans, St. Andrews, N.B. (J. Parsons, personal communication).

We estimated the availability of potential filamentous biota for scallop spat settlement at each of the nine stations (Brand et al. 1980; Minchin 1992; Harvey et al. 1993). During dives made from 17 to 21 July 1992, we recorded the presence or absence of bryozoans, hydroids, rhodophytes, and phaeophyta in 52 randomly placed 0.04-m^2 quadrats at each station. At the same time, we noted the presence or absence of potential predators of larval scallops, such as cerianthid anemones, other anemones (i.e., *Metridium senile*), and tunicates (Langton and Robinson 1990).

Scallop growth rates, in 1992, at stations 1, 2, 3, 5, 6, and 7 were determined by tagging juveniles scallops 15–35 mm in shell height (i.e., 1991 year class) and holding them in cages. Each cage was made of 1 × 1 cm mesh Vexar™ and covered 0.15 m^2 of the bottom. Each scallop was tagged with an identification number glued to its right valve. Following tagging, scallops were held for 48 h in pearl nets, after which dead or stressed scallops (which did not respond to touch) were removed. At each station, 60 juvenile scallops were placed in each cage. The average initial shell height of scallops was 25.3 ± 2.61 mm and they covered ≈20% of the bottom area of each cage, similar to stocking densities used in commercial aquaculture (Parsons and Dadswell 1992). Growth experiments began on 10 June 1992. Shell heights were remeasured on 10 August (after 62 d) and again on 27 October (after 141 d), except at stations 1 and 2 where we were unable to find the cages in October. On 27 October, the scallops were dissected to determine the wet weights of the muscle, gonad, and remaining soft tissues.

Potential predation by fishes was evaluated from stomach contents of fish netted from inside and outside of scallop beds. From 3 July to 20 September 1991, two 1 × 25 m

Fig. 2. Cluster analysis, using euclidean distance and the average linkage method, showing the degree of similarity among the nine stations in Port Daniel Bay in respect to (A) substratum type and (B) the occurrence of bryozoans, hydroids, and the rhodophytes *Ptilota serrata*, and *Phycodrys rubens*. The analysis was based on 52 randomly placed 0.04-m^2 quadrats at each station.



trammel nets were set simultaneously, one near station 1, on bedrock, and one near station 3, on gravel inside the Lighthouse bed. In 1992, an 8 × 45 m gillnet (7.5-cm mesh) was set on consecutive nights from 26 June to 5 August 1992 at stations 1, 3, and 8. Fishing effort was similar at each station during each year. Trammel nets were set 16 times for an average period of 16.4 ± 6.42 h·set^{-1} at station 1 and 16.4 ± 6.12 h·set^{-1} at station 3. Gillnets were set five times for an average of 15.4 ± 1.07, 15.3 ± 1.56, and 15.6 ± 0.96 h·set^{-1} at stations 1, 3, and 8, respectively.

Potential mortality from predators was estimated using scallop tethering experiments at stations 1, 2, and 3 in 1991 and at all nine stations in 1992 (Fig. 1). This technique allowed comparisons among stations and examination of shell fragments indicated predator type. The scallops were tethered at 1-m intervals along a transect line using a 50-cm (4.6-kg test) monofilament line attached through a hole drilled in the auricle of the shell (at the byssal notch). The density of scallops along the transect line was similar to that in the Lighthouse bed. Few scallops (1.3%) broke their tethering lines and we assumed that scallops that were not attached to the lines had been taken by predators.

In 1991, two tethering experiments were conducted, one initiated on 6 August the other on 6 September. Thirty scallops (35 to 45-mm shell height in August and 70 to 80-mm shell height in September) were tethered to each transect and mortalities were noted at 2- to 3-d intervals. Scallops alive on 24 September were left attached to the transects until 9 July 1992. In 1992, 35 scallops (10 scallops 55–69 mm and 25 scallops 70–90 mm) were tethered and the transects were deployed on 17 June and examined weekly until 18 August, with a final observation on 27 October. Because the 1991 trials at stations 1, 2, and 3 were not removed until 9 July 1992, the 1992 transects were placed ≈100 m away from the 1991 transects locations.

In both years, during each observation, one diver recorded the number of predators within a 1-m corridor on each side of the tethering transect lines (60-m^2 sample area in 1991 and 70-m^2 sample area in 1992). A second diver determined whether the scallop was alive or dead, and if dead, the state of the shell (intact, chipped, or crushed). Because observations on different dates along the same transect were not independent, only total mortalities at the end of each experiment were used in comparisons among stations.

Data were examined for heterogeneity of variances and when heterogeneous, were corrected using a $\log(x + 1)$ transformation before applying statistical tests. Means presented are actual means and F- and p-values are for transformed data. Percentages used in correlation analysis were arcsine transformed (Sokal and Rohlf 1981). Scheffé's and Tukey HSD tests were used to compare means if an analysis of variance indicated a significant difference (Day and Quinn 1989). After a significant difference was indicated with a χ^2-test, confidence intervals were calculated for individual observed proportions to determine if they differed from expected proportions using a Bonferroni z statistic (Neu et al. 1974).

Results

Physical environment

During both field seasons, the highest and lowest bottom water temperatures were recorded for stations outside the scallop beds. During August–September 1991, station 2 was significantly warmer than station 1 (12.2 ± 1.2°C and 10.9 ± 2.1°C respectively; ANOVA, $F = 4.54$, df = 2, 72, $p < 0.02$, Scheffé's F-test, $p < 0.05$), but neither differed significantly from station 3 (11.1 ± 1.6°C). In the weekly temperature observations between 26 May and 18 August 1992, differences among the nine stations were not significant (ANOVA $F = 0.58$, df = 8, 71, $p > 0.79$). The means varied from 9.0 ± 4.0°C at station 2 to 5.9 ± 3.8°C at station 7. Mean water temperatures during the tidal cycle did not differ significantly for stations 1, 3, and 8 in July 1992 (ANOVA, $F = 1.03$, df = 2, 190, $p > 0.36$). However, during June–July 1992, temperatures were warmer at station 2 than at stations 1 and 3 (ANOVA, $F = 27.61$, df = 2, 174, $p < 0.001$).

Salinities fluctuated inversely with temperature and ranged from 28.3 ± 1.0‰ at station 2 to 29.4 ± 0.9‰ at station 3. Tides in Port Daniel Bay were semidiurnal and the maximum range recorded was 1.7 m. Current velocities and directions at stations within scallop beds were similar to those at stations outside the beds (Table 1). Within Port

Table 2. Mean number (with standard deviations given in parentheses) of *Placopecten magellanicus* and *Chlamys islandica* spat settling on collectors placed at various stations in Port Daniel Bay in 1991 and 1992.

	Placopten magellanicus		*Chlamys islandica*	
Station	1991	1992	1991	1992
1	1.8 (1.8)		1743.2 (660.9)	
2	1.8 (1.5)	5.5 (3.3)	97.0 (56.6)	1656.5 (650.5)
3	0.4 (0.6)	20.5 (7.6)	360.2 (221.4)	1690.7 (865.2)
5	—	9.7 (5.8)	—	2527.7 (1443.4)
6	—	10.7 (3.1)	—	1904.3 (531.0)
7	—	3.7 (2.0)	—	351.3 (180.9)
8	—	4.5 (1.1)	—	260.3 (112.0)

Table 3. Mean daily increase (with standard deviations given in parentheses) in shell height and weight (g) of the muscle and viscera for *Placopecten magellanicus* maintained in cages on the bottom at six stations during 1992. Means with the same letter are not significantly different ($p > 0.05$, Scheffé's and Tukey HSD tests).

	10 June – 10 August		10 August – 27 October		10 June – 27 October		
Station	*n*	Increment·day^{-1} (mm)	*n*	Increment·day^{-1} (mm)	*n*	Muscle wt. (g)	Viscera wt. (g)
1	36	0.126 (0.017)*b*					
2	39	0.133 (0.018)*b*					
3	52	0.110 (0.024)*a*	37	0.114 (0.012)*ab*	47	0.94 (0.14)*b*	2.07 (0.33)*b*
5	48	0.111 (0.016)*a*	38	0.117 (0.013)*ab*	43	0.63 (0.14)*a*	1.66 (0.35)*a*
6	55	0.101 (0.016)*a*	39	0.108 (0.017)*a*	42	0.89 (0.20)*b*	2.23 (0.57)*b*
7	42	0.172 (0.021)*c*	38	0.125 (0.021)*b*	44	1.15 (0.22)*c*	2.66 (0.54)*c*

Daniel Bay mean current velocities ranged from 6.3 ± 3.8 to 10.2 ± 6.8 cm·s^{-1} and the mean residual current direction was to the south-southwesterly ranging from 170 ± 75.9° to 196 ± 81.3°.

The highest scallop densities were found at stations 3 and 8 where the substratum was predominantly (>80%) gravel (Table 1). Station 9 also had a predominantly gravel substratum yet scallop density was lower than in the two scallop beds, but higher than the remaining stations. Most stations contained several types of substratum. Cluster analysis, using euclidean distance (Krebs 1989) based on substratum type, showed that station 8, inside the Anse à la Barbe scallop bed, was most similar to stations 5 and 4, outside the scallop beds (probably because of similar proportions of sand and cobble at all three of these stations), and next most similar to station 3, inside the Lighthouse bed (Table 1, Fig. 2). Stations 2 and 7 were relatively similar, whereas stations 1, 6, and 9 were relatively unique (Fig. 2).

Settlement

The numbers of *P. magellanicus* spat settling on collectors at stations 1, 2, and 3 in 1991 were similar (ANOVA, $F = 1.66$, df = 2, 13, $p > 0.23$; Table 2). However, settlement varied among the six stations studied in 1992 and was highest at station 3, inside the Lighthouse bed (ANOVA, $F = 6.64$, df = 5, 30, $p < 0.001$; Table 2). Spat counts were higher for collectors set in 1992 than for those set in 1991 (t-test, df = 1, $p < 0.02$). This difference between years probably resulted from postsettlement mortality, as 1991 collectors were in the water for 10 months compared with 2 months for the 1992 collectors. However, no evidence of mortality, such as shell fragments, was observed in the collector bags. *Placopecten magellanicus* spat size ranged from 4.1 ± 2.6 to 6.2 ± 3.8 mm in October, 1992. In both years *C. islandica* settled in much greater numbers than *P. magellanicus* (Table 2).

Cluster analysis using euclidean distance and the average linkage method (Krebs 1989), applied to filamentous organism occurrence at the nine stations, showed high similarity between stations 3 and 8 inside scallop beds (Fig. 2). Filamentous fauna, bryozoans (present in 33.9% of all quadrats), and hydroids (31.9%), and the rhodophytes *Ptilota serrata* (18.7%) and *Phycodrys rubens* (27.6%) were the most common from all station data combined. Bryozoans and algae had similar abundance inside and outside beds. Hydroids were found more often within scallop beds ($\chi^2 = 81.2$, df = 8, $p < 0.001$) and were present in 39 of 52 quadrats at both stations 3 and 8 compared with abundances ranging from 2 to 21 (quadrats) at the stations outside of the scallop beds.

Table 4. Fish species collected in Port Daniel Bay during 1991 and 1992 that contained *Placopecten magellanicus* in their stomach contents.

		Inside scallop beds			Outside scallop beds		
Fish species	Year	*n*	Ingested scallops	%	*n*	Ingested scallops	%
Pseudopleuronectes americanus	1991	53	5	9.4	29	0	0
	1992	80	3	3.8	25	0	0
Myoxocephalus scorpius	1991	14	0	0	19	0	0
	1992	10	1	10	5	0	0
Anarhichas lupus	1991	0	0	0	1	1	100
	1992	1	1	100	0	0	0

Growth rates

Growth over 4 months varied markedly among stations (10 June – 10 August 1992; ANCOVA, $r^2 = 0.81$, $F = 70.61, 867.93$, $n = 279$, df = 5, 1, $p < 0.001$; for 10 August – 27 October 1992; ANCOVA, $r^2 = 0.78$, $F = 5.34, 345.50$, $n = 142$, df = 3, 1, $p < 0.001$) (Table 3). Average growth rate, for all stations, was 0.12 ± 0.03 mm·d^{-1} from 10 June to 10 August and 0.6 ± 0.01 mm·d^{-1} for 10 June to 27 October 1992. Average growth rate ranged from 0.172 mm·d^{-1} at station 7 to 0.101 mm·d^{-1} at station 6, 2.5 km apart and at similar depths (18 and 21 m, respectively), and was below average at station 3, within the Lighthouse bed. During the 141-d growth trials, tag loss ranged from 0 to 20%. Scallops eaten by small starfish (<12%) were excluded from the analyses.

Growth rates over an 11-month period (6 September 1991 – 9 July 1992) for larger scallops (1991 tethering experiment; >75 mm) were similar at stations 1 (shell height, 0.029 ± 0.002 mm·d^{-1}), 2 (0.032 ± 0.010 mm·d^{-1}) and 3 (0.034 ± 0.011 mm·d^{-1}) (ANOVA, $F = 0.287$, df = 2, 29, $p > 0.74$); (two-way ANOVA (station, sex), muscle, $p > 0.56$; gonad, $p > 0.50$; remaining viscera, $p > 0.21$).

Predation

Placopecten magellanicus remains were found in stomachs of 3 of 22 fish species trapped in nets set during 1991 and 1992. While small scallops were completely ingested, only the soft tissue of large scallops were found in fish stomachs. Scallop tissue eaten by fishes was usually fresh and easily identified. Of the winter flounder, *Pseudopleuronectes americanus*, collected inside scallops beds in 1991 (fork length 34.3 ± 5.09 cm) and 1992 (29.7 ± 0.58 cm), 9.4 and 3.8%, respectively, had eaten scallops (Table 4). *Placopecten magellanicus* was found in one of 48 shorthorn sculpin *Myoxocephalus scorpius* (32 cm fork length) collected inside the Lighthouse bed in 1992. Two Atlantic wolffish (*Anarhichas lupus*; 79.3 ± 8.13 cm) were collected, one inside and one outside of scallop beds, and both contained scallops.

Tethering experiments initiated in 1991 indicated that predation was significantly less at stations inside than outside scallop beds (Table 5, Fig. 3). Predation on large scallops (70 to 90-mm shell height) was also greater outside of the scallop beds in 1992, but predation on intermediate sized scallops (55–69 mm) was not significantly different ($\chi^2 = 6.0$, df = 8, $p > 0.65$; Table 5, Fig. 3). After 4 months (27 October 1992), the lowest mortality was still at station 3, although the rates no longer varied significantly among stations ($\chi^2 = 9.5$, df = 6, $p > 0.19$; Table 5). The losses of the tethering transects at stations 1 and 8 decreased the power of the χ^2 test to detect differences in the latter test.

Mean densities of each predator species varied significantly among substrata (nested ANOVA; 1991, $F = 0.03, 13.36$, df = 2, 15, $p < 0.001$; 1992, $F = 0.46, 16.13$, df = 3, 28, $p < 0.001$) (Table 6), with asteroids most abundant on gravel and cobble substrata. The sea star *Leptasterias polaris* was the only scallop predator which was significantly correlated with scallop density ($r^2 = 0.46$, Bonferroni t-test = 4.06, df = 1, $p < 0.04$). Predator densities varied considerably among stations even when the substratum was similar. In 1992, cerianthid anemones were absent from all stations and sea anemones and tunicates, which might prey on scallop larvae, were only observed in <5% of the quadrats at stations 3 and 9.

Percent mortality of tethered small scallops, 35–45 mm, at each station was positively correlated with mean densities of rock crab, *Cancer irroratus*, (Pearson's $r = 0.88$), and winter flounder, *Pseudopleuronectes americanus* (Pearson's $r = 0.94$), though not significantly owing to the low degrees of freedom. Loss of intermediate sized scallops, 55–69 mm, was not correlated with any species. Percent mortality of large 70- to 90-mm scallops at each station was significantly correlated with lobster mean density (Pearson's $r = 0.78$, Bonferroni t-test = 6.18, df = 7, $p < 0.02$; Fig. 4) in 1992 and positively correlated, though not significantly in 1991 ($r = 0.71$).

Examination of scallops shells that remained attached to tethering lines indicated that decapods were the primary predator at most stations (Fig. 5). The scallop's shell was not damaged by asteroid predators. The shells of scallops eaten by decapods were either chipped along the margin (76%) or occasionally completely crushed (24%). Some scallops were missing. This may be attributable to decapods because they transport prey to their dens (Moody and Steneck 1993).

On a number of occasions we observed predators attacking and eating scallops. On 12 September 1991, a lobster *Homarus americanus* (80–90 mm in carapace length), was observed grasping at 76-mm scallop with its second chelae while the chelate dactyls were used to chip away the edge

of the shell. Subsequently, the lobster pinched and tore away the mantle tissue, causing the valves to open. During this attack, the lobster ate, it transported the scallop ≈60 m along the bottom. *Leptasterias polaris*, were encountered attacking scallops on 10 occasions. Usually attacks were by a single seastar but in two instances two *L. polaris* were feeding on the same scallop and in another a *L. polaris* and an *Asterias vulgaris* were feeding on the same scallop. The spiny sunstar *Crossaster papposus* was observed feeding on a scallop on three occasions.

Discussion

Physical hydrographic conditions did not appear to explain where scallop beds were located, at the scale of kilometers, within Port Daniel Bay because conditions within scallops beds were similar to those at stations in the surrounding areas where scallops were scarce. Mean temperature varied by <3.0°C and mean salinity by <1.0‰ among stations. Mean residual current flow was generally parallel to the coastline (south-southwesterly) and varied by <30° among stations. Mean current velocity varied by <4 $cm \cdot s^{-1}$ among stations and was below the thresholds inhibiting feeding (≈10 $cm \cdot s^{-1}$; Wildish et al. 1987; Wildish and Saulnier 1992) and causing passive transport of scallops (11–37 $cm \cdot s^{-1}$; Grant et al. 1993).

A consistent feature of scallop aggregations (beds), observed in the present and previous studies, is an association with gravel substratum (Brand 1991; Thouzeau et al. 1991*a*; Stokesbury and Himmelman 1993). However, gravel bottoms do not always support scallop aggregations. In our study, scallop densities were low at stations 5 and 9, and both had a high proportion of gravel substratum. This suggests that other factors are also required for the creation and maintenance of scallop beds.

Higher scallop spat settlement did not always occur within scallop beds compared with adjacent areas. The intensity of spat settlement was greatest at station 3 in the Lighthouse bed in 1992, but only intermediate at station 8 in the Anse à la Barbe bed and extremely low at station 3 in 1991. In the Port Daniel region, larvae of *C. islandica* settled in far greater numbers than larvae of *P. magellanicus* yet adult *C. islandica* were rare. Evidence that high settlement is not critical for scallop beds to develop was previously reported from studies of *P. magellanicus* in Passamaquoddy Bay, New Brunswick (Robinson et al. 1992), and from studies of *Pecten maximus* on the Irish coast (Minchin 1992). It is dubious whether the intensity of spat settlement explains where scallop beds become established (Brand 1991). Marked interannual fluctuations in settlement, as we observed between 1991 and 1992, have been reported previously and may explain fluctuations in the strength of annual cohorts (Caddy 1989).

Filamentous fauna and flora, which are some of the preferred substrata for scallop settlement (Brand et al. 1980; Minchin 1992; Harvey et al. 1993), were associated with scallop beds in Port Daniel Bay. Hydroids were significantly more abundant inside scallop beds than in adjacent areas. Although scallop settlement within collectors has been positively correlated with larval scallop density in the water column (S. Robinson, Department of Fisheries and Oceans, St. Andrews, N.B., personal communication) collectors may underestimate spat settlement densities in areas with suitable settlement substratum (Harvey et al. 1993). Therefore, settlement may be greater in specific locations (e.g., scallop beds) because of the increased availability of settlement substrata even though densities of scallop larvae in the water column may be evenly or randomly distributed over a large area. Settlement in scallop beds may further be enhanced because these filamentous organisms often colonize the shells of adult scallops (personal observations).

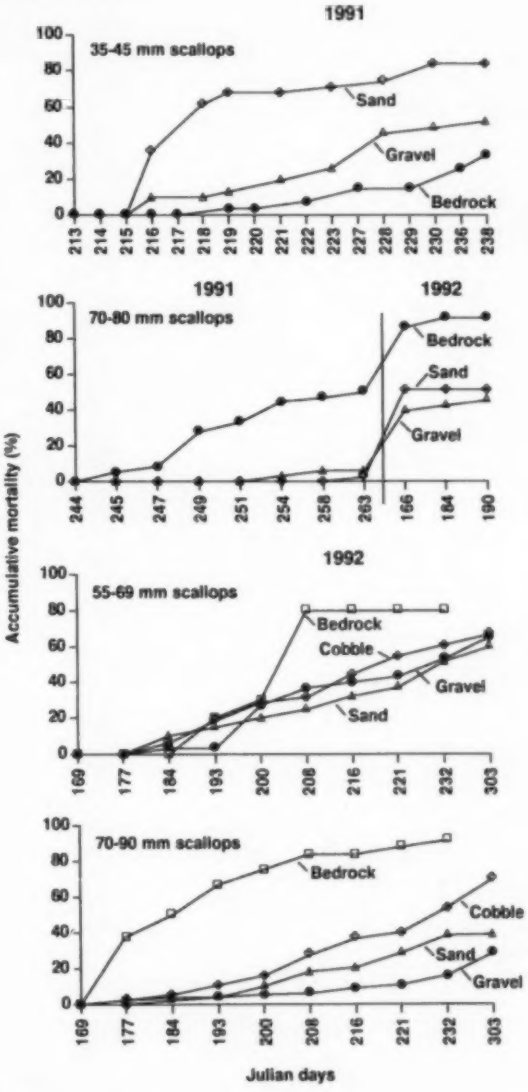


Fig. 3. Cumulative loss of tethered *Placopecten magellanicus*, for 35- to 45-mm scallops between 6 and 26 August 1991, 70- to 80-mm scallops between 6 September 1991 and 9 July 1992, and 55- to 69-mm and 70- to 90-mm scallops between 17 June and 27 October 1992.

Table 5. Chi-square comparisons of the percentage mortality of tethered *Placopecten magellanicus* of various sizes placed at different stations during 1991 and 1992. Individual observations that were significantly different ($p < 0.05$, using a Bonferroni z statistic) from expected values are indicated with an asterisk.

(A) 1992–1992 experiment.

	35- to 45-mm scallops (6–26 Aug. 1991)		70- to 80-mm scallops (6–24 Sept. 1991)		70- to 80-mm scallops (Sept. 1991 – July 1992)	
Station	*n*	%	*n*	%	*n*	%
1	27	33.3	36	50.0*	36	91.7
2	31	83.9	35	2.9*	35	51.4
3	31	51.6	33	6.1*	33	45.5
	$\chi^2 = 6.7$	$p < 0.03$	$\chi^2 = 21.4$	$p < 0.001$	$\chi^2 = 7.0$	$p < 0.03$

(B) 1992 experiment.

	55- to 69-mm scallops (17 June – 18 Aug. 1992)		70- 90-mm scallops (17 June – 18 Aug. 1992)		55- to 90-mm scallops (17 June – 27 Oct. 1992)	
Station	*n*	%	*n*	%	*n*	%
1	10	80.0	24	91.7*		
2	10	60.0	23	17.4	33	45.5
3	10	30.0	27	7.4*	37	37.8
4	10	40.0	23	47.8	33	60.6
5	11	90.9	26	65.4	37	81.1
6	10	50.0	25	48.0	35	65.7
7	7	42.9	25	60.0	32	56.3
8	10	50.0	24	25.0		
9	10	80.0	25	16.0*	35	40.0
	$\chi^2 = 6.0$	$p > 0.65$	$\chi^2 = 36.6$	$p < 0.001$	$\chi^2 = 9.5$	$p > 0.19$

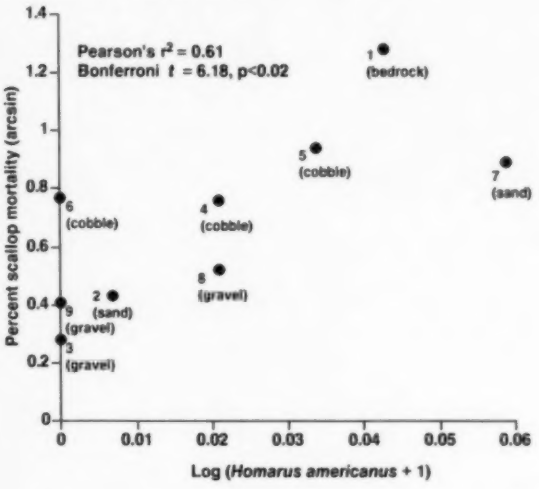
Scallop beds were not located in areas where conditions enhanced scallop growth. Growth rates at station 3, within the Lighthouse bed, were intermediate to those occurring at stations in adjacent areas. Further, growth rates for scallops at station 3 in the Lighthouse bed were similar to those at stations 1 and 2 over a 1 year period. Growth rate of *P. magellanicus* varies substantially over its geographic range and these differences are usually attributed to temperature and food availability (MacDonald and Thompson 1985*a*, 1985*b*, 1986*a*; Schick et al. 1988). *Placopecten magellanicus* growth rates on the substratum in Port Daniel Bay were similar to those found in suspended culture in the same area (Côté et al. 1993) and on both the substratum and in suspended culture in other locations (Parsons and Dadswell 1992).

Whether predation by fish is a major determining factor for scallop aggregations is unclear. Our analyses of stomach contents indicates that common fish predators of *P. magellanicus* in the Port Daniel Bay region were winter flounder and Atlantic wolffish. American plaice, *Hippogossoides platessoides*, also feed on *P. magellanicus* (Naidu and Meron 1986). High abundance of flounders, which occasionally feed on scallops, might affect scallop density. Atlantic wolffish stays in given areas for prolonged periods (Scott and Scott 1988) and preferentially preys on scallops (Nelson and Ross 1992) and may influence *P. magellanicus* density and spatial distribution in those areas.

Fish predation may increase when scallops are stressed, such as when there are strong temperature fluctuations (Dickie and Medcof 1963). In the Lighthouse bed on 12 September 1991, we observed dead scallops (≈7% of the population) showing no signs of damage to their shells and no associated increase in asteroid density. On 27–29 July 1992, many scallops also appeared to be weak; their valves were gaped, they responded slowly when touched, their mantles were retracted from the edge of the shell and some contained sand in the mantle cavity. Shortly following these dates the intact soft tissue of scallops appeared in the stomachs of winter flounder. Intact scallop tissues have also been observed in large numbers of cod *Gadus morhua* caught during June and July 1993 on Georges Bank (M. Strong, Department of Fisheries and Oceans, St. Andrews, N.B., personal communication).

Decapods appeared to inflict considerable mortality on both small and large scallops. Increased losses of tethered scallops were correlated with densities of rock crab and lobster whereas losses from asteroid predation appeared to be similar at most stations. Highest mortalities of small tethered scallops (35–45 mm in shell height) were on sandy bottoms where rock crabs were most abundant, and

Fig. 4. The correlation between the percent mortality of tethered 70- to 90-mm *Placopecten magellanicus* and the mean density of *Homarus americanus* (individuals·10 m^{-2}) at the nine stations in Port Daniel Bay from 17 June to 18 August 1992.



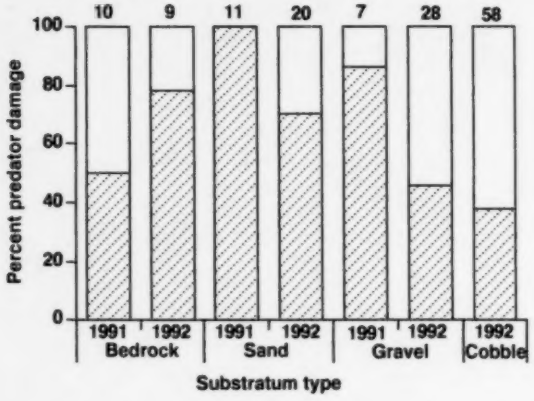
shell remains indicated that most mortalities were from predation by decapods. Mortality of medium size scallops (55–69 mm) was not correlated with the abundance of any predator. In contrast, mortality of large scallops (>70 mm) was correlated with abundance of lobsters. A high proportion of the dead large scallops had broken shells, similarly, indicating decapod predation. Because our tethering trials restricted the potential escape response (i.e., movement) of scallops, percentage losses may overestimate potential mortality (Aronson 1992; Pohle et al. 1991; Maeda-Martinez et al. 1993).

Our data indicating that lobsters (size range ≈50- to 120-mm carapace length) preferentially preyed on large scallops (>70 mm) contradicts earlier reports that lobsters (mean carapace length ranged from 77.2 mm ± 12.2 to 94.5 ± 11.9 mm) select 20- to 50-mm individuals and do not attack >70-mm scallops (Elner and Jamieson 1979; Jamieson et al. 1982). Jamieson et al. (1982) observed cannibalism among lobsters maintained in corrals in the field in the presence of 80 to 110-mm scallops. Juanes (1992) states that decapods select smaller scallops because they are easier to crush and, therefore, present low risk of claw damage. Claw damage increases with shell height because the force of crusher chelae increases linearly with size whereas the strength of the scallop shell increases exponentially with size (Elner and Campbell 1981). Elner and Jamieson (1979) and Jamieson et al. (1982) observed that lobsters could use either chipping or crushing techniques but stated that the chipping technique is rarely successful. However, Elner and Jamieson (1979) starved their lobsters for 2 d prior to the experiment, which possibly reduced the amount of handling time the lobsters were willing to spend on each prey. Crushing the scallop probably exposes the soft tissue more rapidly than chipping the shell edge. Jamieson et al. (1982) greatly increased the densities of lobsters within their corrals

Table 6. Mean (with standard deviations given in parentheses) density (individuals·10 m^{-2}) of predator species at the nine stations during 1991 and 1992.

Predator	Year	Station 1	2	3	4	5	6	7	8	9	*p*
Homarus americanus	1991	0.19 (0.17)	0.03 (0.06)	0.10 (0.17)							0.008
	1992	0.11 (0.10)	0.02 (0.05)	0	0.05 (0.11)	0.09 (0.15)	0	0.16 (0.21)	0.05 (0.11)	0	0.034
Cancer irroratus	1991	0.02 (0.05)	0.56 (0.43)	0.19 (0.21)							0.001
	1992	0.02 (0.05)	1.27 (1.27)	0.18 (0.18)	0.75 (1.19)	0.01 (0.10)	0.18 (0.21)	0.80 (1.22)	0.13 (0.25)	0.07 (0.20)	0.005
Hyas ssp.	1992	0	0	0.02 (0.05)	0.23 (0.60)	0.02 (0.05)	0.07 (0.08)	0	0.05 (0.07)	0	0.380
Leptasterias polaris	1991	0.27 (0.23)	0	0.32 (0.37)							0.001
	1992	0.18 (0.23)	0	0.82 (0.59)	0.30 (0.31)	1.50 (0.60)	0.43 (0.29)	0.02 (0.05)	1.54 (0.74)	0.71 (0.60)	0.001
Asterias vulgaris	1991	0.10 (0.15)	0.06 (0.10)	0.32 (0.37)							0.630
	1992	0.04 (0.07)	0.13 (0.23)	0.32 (0.23)	0.75 (1.11)	0.93 (0.45)	0.30 (0.58)	0.05 (0.15)	0.45 (0.49)	0.14 (0.20)	0.003
Crossaster papposus	1991	0	0	0.03 (0.06)							0.041
	1992	0.18 (0.15)	0	0.20 (0.19)	0.07 (0.11)	0.18 (0.17)	0.16 (0.18)	0	0.18 (0.20)	0	0.003
Pseudopleuronectes americanus	1991	0.04 (0.09)	0.10 (0.13)	0.02 (0.05)							0.032
	1992	0.02 (0.05)	0.13 (0.18)	0.05 (0.07)	0.05 (0.11)	0.04 (0.07)	0.04 (0.07)	0.21 (0.20)	0.04 (0.07)	0.05 (0.07)	0.020
Cottidea	1992	0	0.02 (0.05)	0.02 (0.05)	0	0	0.04 (0.07)	0.02 (0.05)	0.04 (0.07)	0	0.470

Fig. 5. Percentage of *Placopecten magellanicus* (all sizes) damaged by decapods and asteroids in the tethering experiments in 1991 and 1992 conducted on four different substrata in Port Daniel Bay. Decapod predators are represented by the shaded portion of the bar and asteroid predators by the open portion, the numbers associated with each bar indicates the number of scallops autopsied. The percentages were calculated from the final observation of each transect line.



(maximum density 1.4 lobsters·m^{-2}) thereby reducing the available shelter and possibly increasing the rate of intraspecific contact and cannibalism. Thus, the experimental design of both these studies may have influenced lobsters feeding habits. Our data showed that both rock crabs and lobsters usually chipped the margin of the shell rather than crushing it. This technique allows lobsters to prey on larger scallops without increasing the risk of claw damage. Large scallops were not immune to predation by lobsters. In areas with high lobster density, such as stations 1, 5, and 7 in our study, scallops may not be able to establish aggregations because lobster predation rate is too high.

In conclusion, our studies in Port Daniel Bay indicated that the spatial distribution of scallop beds did not appear to be associated with hydrographic conditions (current velocity, temperature, and salinity), asteroid predation, or areas of greater growth. However, they were associated with the presence of gravel substratum, low decapod and fish predation, and presence of filamentous flora and fauna (particularly hydroids).

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Groundwater contribution to the water and chemical budgets of Williams Lake, Minnesota, 1980–1991

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Abstract: Groundwater seepage was the largest annual flux of water into (58–76%) and out of (73–83%) Williams Lake during a 12-year study, during which the entire volume of the lake was replaced four times. The only other water fluxes to and from the lake, which has no surface-water inlet or outlet, were atmospheric precipitation and evaporation. Nearly all of the annual input of calcium, magnesium, sodium, potassium, chloride, sulfate, and silica was provided by groundwater. Although much of the calcium and most of the silica input was retained in the lake, this retention did not result in increased chemical mass in the lake water mass because biologically mediated removal of calcium and silica to the sediments equaled or exceeded loss by lake seepage to groundwater. Groundwater represented as much as one-half the annual hydrological input of phosphorus and nitrogen; the remainder was supplied by atmospheric precipitation. From about 70 to 90% of the annual input of phosphorus and nitrogen was retained in the lake. Although water and chemical fluxes varied from year to year, interaction of the lake with groundwater determined the hydrological and chemical characteristics of Williams Lake.

Résumé : Les infiltrations d'eau souterraine constituaient le principal apport d'eau (58–76%) et la principale perte d'eau (73–83%) du lac Williams tout au long d'une période d'étude de 12 ans au cours de laquelle le volume entier du lac a été remplacé quatre fois. Les seuls autres apports et pertes d'eau de ce lac, qui n'a ni tributaire ni exutoire d'eau de surface, ont été les précipitations et l'évaporation. Les apports annuels en calcium, en magnésium, en sodium, en potassium, en chlore, en sulfates et en silicium ont été dus presque exclusivement à l'eau souterraine. Même si une grande partie du calcium et presque tout le silicium arrivés dans le lac y sont restés, cette rétention n'a pas augmenté la masse des substances chimiques dans la masse d'eau puisque la fixation du calcium et du silicium dans les sédiments, par le biais de processus biologiques, a égalé ou dépassé les pertes dues aux infiltrations dans la nappe d'eau souterraine. L'apport hydrologique annuel de phosphore et d'azote était également dû, pour jusqu'à la moitié du total, à l'eau souterraine; le reste est venu des précipitations. La proportion de l'apport de phosphore et d'azote retenus dans le lac a oscillé entre 70 et 90%. Même si les apports et les pertes d'eau et de substances chimiques ont varié d'une année à l'autre, les interactions entre les eaux du lac et les eaux souterraines ont déterminé les caractéristiques hydrologiques et chimiques du lac Williams.
[Traduit par la Rédaction]

Introduction

Determination of hydrologically controlled transport of major ions and nutrients into and out of watersheds and lakes has provided valuable information on ecosystem processes in several studies (Schindler et al. 1976; Likens 1985; Jeffries et al. 1988). It is recognized increasingly that groundwater plays a part in the interaction between surface waters and their watersheds (Hynes 1975; Likens 1984). Yet, comprehensive studies of water and chemical balances of lakes that include measurement of the groundwater component are rare (Malueg et al. 1975; Schindler et al. 1976). More often, in the few cases where groundwater is recognized as part of water or chemical budgets of lakes, its net contribution has been determined by the difference between measured gains and losses (Likens 1985; Jeffries et al. 1988; Staubitz and Zarriello 1989; Krabbenhoft et al. 1990).

Some of the studies of lakes in which groundwater was measured directly found groundwater contributions to water and chemical fluxes to be negligible (Malueg et al. 1975; Schindler et al. 1976). However, other studies indicate that groundwater can supply phosphorus (Brock et al. 1982; Shaw et al. 1990), nitrogen (Brock et al. 1982), silica (Hurley et al. 1985), or major ions (Schwartz and Gallup 1978; Kenoyer and Anderson 1989). Processes occurring along the groundwater flow path prior to discharging into lakes can have an effect on chemical characteristics of the

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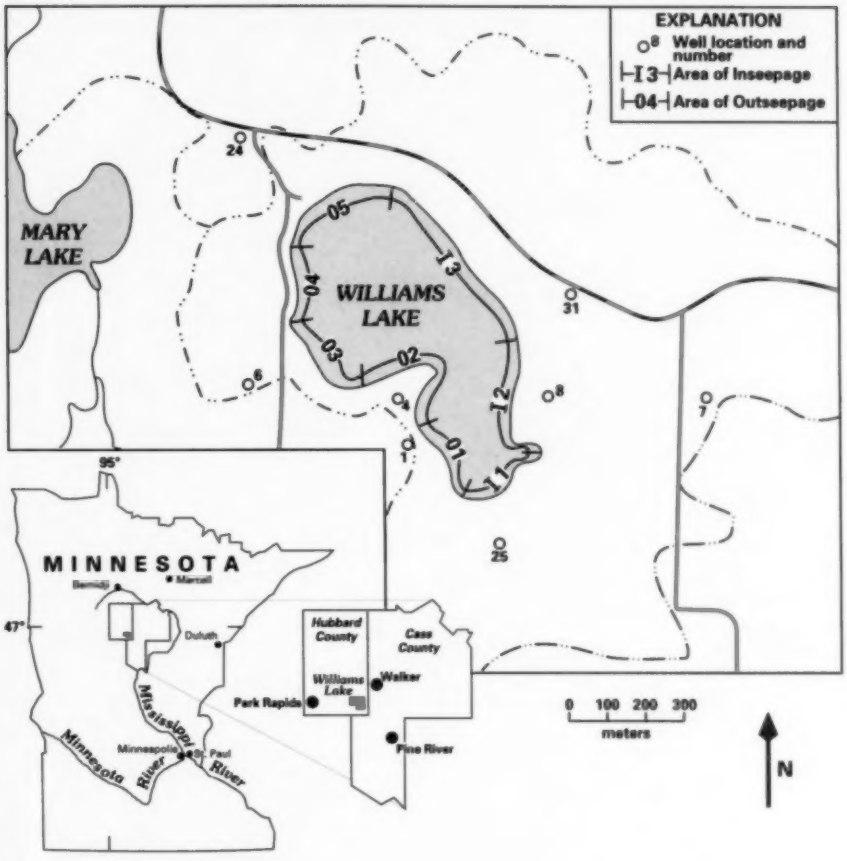
Fig. 1. Location of wells from which water was collected for chemical analysis, and areas of groundwater seepage to and from the lake.



receiving lakes, as indicated in studies of acid deposition (Anderson and Bowser 1986; Craig and Johnston 1988). While the number of studies examining groundwater seepage to lakes is increasing, there are few studies documenting loss of water and chemicals from lakes by seepage to groundwater (LaBaugh 1986; Kenoyer and Anderson 1989; Sacks et al. 1992). Studies directly measuring both chemical groundwater input to lakes and chemical output to groundwater from lakes over a period of many years are virtually nonexistent. The question remains: how important is groundwater seepage to lakes and lake seepage to groundwater in determining the hydrological and chemical characteristics of lakes, particularly those without interaction with channelized surface water?

Studies at Williams Lake, Minnesota, during 1980–1991 have included direct measurement of the interaction between groundwater and the lake. This lake was chosen for study because of its perceived linkage with groundwater and because it had no surface water inlets or outlets (Siegel and Winter 1980). Hydrological research at this site also has included investigation of rainfall and evaporation (Sturrock et al. 1992). Chemical characteristics of the lake and groundwater (LaBaugh 1988, 1991) and atmospheric deposition were evaluated concurrently during a 12-year period. Availability of hydrological and chemical data enabled determination of the relative contributions to water and chemical budgets of the lake from seepage to and from groundwater. The purpose of this report is to present the analysis of the relative contribution of groundwater to major-ion and nutrient balances for Williams Lake during a period of variable hydrological conditions.

Site description

Williams Lake is located in Hubbard County, north-central Minnesota (Fig. 1). It is 36 ha in surface area and 9.8 m deep and is underlain by thick, calcareous glacial drift characteristic of ice-contact deposits (Siegel and Winter 1980). Mixed deciduous and coniferous forest covers most of the lake's 227-ha watershed, which contains a few permanent and several seasonal residences. Climate in this area is characterized as continental. The lake is in an area where annual average lake evaporation is approximately equal to annual average precipitation (Sturrock et al. 1992). Ice covers the lake from mid-November to mid-April. Water in the lake is thermally stratified in summer and

winter; isothermal conditions are least persistent in spring and of longer duration in the fall. Other details about the lake and its watershed are provided by Siegel and Winter (1980), LaBaugh et al. (1981) and Sturrock et al. (1992).

Materials and methods

Hydrological instrumentation

Rainfall was measured using a recording tipping-bucket rain gage located on land near the lakeshore and operated during periods when the lake was not ice covered (Sturrock et al. 1984; Rosenberry et al. 1988). During months when the tipping-bucket gage was not operating, usually November to March, atmospheric precipitation was estimated as a weighted average of data from three nearby U.S. National Weather Service stations located at the Pine River Dam, the Walker ranger station, and the town of Park Rapids. Comparability of precipitation measured at the study site and estimated from the National Weather Service data is given by Siegel and Winter (1980).

Evaporation was measured using the mass-transfer and energy-budget methods during the periods when the lake was not ice covered. Measurements were made on land in the vicinity of the tipping-bucket rain gage for incoming short- and long-wave radiation, and on a raft near the middle of the lake for measurement of wind profiles, atmospheric temperature, surface-water temperature, and vapor pressure. Details of this instrumentation and related auxiliary instrumentation are presented by Sturrock et al. (1992).

Water-table wells (3 and 5 cm diameter) were installed in the vicinity of the lake by drilling with power and hand augers. The wells were used to determine hydraulic gradients between groundwater and the lake. Wells were constructed using either steel or PVC casing with wire-wound well screens of similar material. Details of well installation are given by Siegel and Winter (1980). Water levels in the wells were determined by use of a calibrated steel tape by measuring from a fixed measurement point on the well casing. Measurements were made weekly in spring, biweekly during summer and fall, and monthly during winter for each year of the study. Altitudes of the measurement point of each well were leveled to a common datum.

Change in elevation of the surface of the lake was measured using a recording gage. A stilling-well-type gage and A-30 recorder were used for 1980–1985. Beginning in 1985, a bubble gage was used in conjunction with a paper-tape punch recorder. Lake areas for given stages were determined by planimetry of a 0.6-m contour interval map of the lake bottom provided by the Minnesota Department of Natural Resources.

Water sample collection for chemical analyses

Bulk precipitation was collected for chemical analysis of phosphorus in the vicinity of the tipping-bucket rain gage by use of a collector similar in design to that of Likens (1972) from 1980 to 1981. A wet–dry collector was used to collect water from the wet collector for analysis of phosphorus beginning in 1981 and total Kjeldahl nitrogen and silica beginning in 1985. In 1989, large bulk collectors consisting of 132-L plastic buckets lined with polyethylene also were used to collect atmospheric deposition for chemical analysis of major ions and silica. Water was collected from precipitation collectors at approximately 2-week intervals when the lake was not ice covered.

From the beginning of the study through 1991, 307 samples of groundwater were collected from a total of 42 wells. Collection was on a regular basis for eight wells and an irregular basis for the remainder. These data indicated that some of the wells in close proximity to the lake from which water was collected on a regular basis were representative of areas of groundwater seepage to and from the lake. LaBaugh (1991) provides a summary of chemical characteristics of all wells at the study site.

Water was collected on a monthly basis during the period when the lake was not ice covered, usually from April to November. Water-table wells were selected on the basis of their location with respect to seepage to and from the lake. A peristaltic pump was used to collect water from wells that had a water level within 10 m of land surface. Collection by bailer was done for water-table wells whose water level was deeper than 10 m below land surface. At the beginning of the study, field analysis of water collected from wells indicated water representative of groundwater adjacent to the well was obtained after water representing three times the volume standing in the well casing had been removed. Subsequently, samples were collected after a volume of water equal to three times the volume standing in the well casing had been removed, either by pumping or bailing. Determinations of temperature, pH, and specific conductance were made on water collected from the wells at the time of sample collection. Groundwater samples were collected occasionally at the lakeshore line by using a portable, small-diameter sampling device. This device is described by Winter et al. (1988).

Water was collected from the lake approximately every 2 weeks when the lake was not ice covered and approximately monthly when the lake was covered with ice. Samples for major-ion analysis were collected using a PVC van Dorn bottle at 1 m below lake surface and 1 m above lake bottom in the vicinity of the deepest part of the lake. Water for nutrient analysis was obtained by combining samples collected from the lake surface and at 1-m intervals through the photic zone. The photic zone was defined by doubling the value for Secchi disk transparency, which was assumed to represent the depth to which 1% of surface light penetrated the lake. Measurements of temperature, pH, specific conductance, and dissolved oxygen were made at the surface and at 1-m intervals to the lake bottom with a multiparameter probe coincident with sample collection from the lake.

Chemical analyses

Water obtained for chemical analyses was not filtered for analysis of pH, specific conductance, total alkalinity, total phosphorus, and total Kjeldahl nitrogen. Water was filtered on site using a 0.45-μm membrane filter for analysis of calcium, magnesium, sodium, potassium, chloride, sulfate, silica, soluble reactive phosphorus, nitrate and nitrite nitrogen, and ammonia nitrogen. Water to be used for major-cation analysis was preserved with 1 mL of nitric acid per 250 mL of sample. Water to be used for nutrient analyses was preserved with mercuric chloride. Water collected for

Table 1. Annual water budgets for Williams Lake, 1980–1991. All values for water volume are in thousands of cubic meters.

	Inputs				Losses				
Year	Precipitation	Percent total input	Groundwater seepage to lake	Percent total input	Evaporation	Percent total output	Lake seepage to groundwater	Percent total output	Change in storage
1980	207	33	429	67	163	19	675	81	−72
1981	290	42	397	58	150	18	669	82	33
1982	271	41	394	59	144	17	688	83	14
1983	235	36	414	64	186	21	684	79	8
1984	230	35	437	65	215	24	696	76	−27
1985	302	42	421	58	193	21	708	79	104
1986	245	35	455	65	201	21	738	79	20
1987	231	33	469	67	213	22	734	78	−97
1988	190	29	465	71	242	27	672	73	−87
1989	183	31	416	69	199	23	672	77	−6
1990	125	24	391	76	180	21	667	79	−89
1991	196	33	396	67	192	24	597	76	−42

Note: Values for each component of the water budget were subject to uncertainty in measurement. Estimates of their uncertainty were 5% for atmospheric deposition, 50% for groundwater, 10% for evaporation, and 5% for change in storage.

pH, specific conductance, and total alkalinity had no preservative added to it.

After collection, all water samples were stored in ice (usually for a day) and then shipped in ice to the U.S. Geological Survey national laboratories for subsequent analysis. Prior to 1981 analyses were done at the laboratory in Atlanta, Ga., and after 1981 at the laboratory in Arvada, Colo. Analytical methods used for chemical analyses are described by Fishman and Friedman (1985).

Calculation of water budgets

Lake-stage data and the 0.6-m contour interval map of the lake bottom were used to determine lake surface area and volume for use in subsequent calculations. Area of each 0.6-m depth interval was determined by planimetry. Volume between depth intervals was determined using Hutchinson's (1957) method of calculation. The contour map was made when the lake stage was 421.4 m, and during the study lake stage exceeded 421.4 m. At lake-surface altitudes less than 421.4 m the differences in volume between 421.4 and 420.8 m were used to determine volumes at individual values of stage by interpolation. For altitudes of lake stage greater than 421.4 m, volume was determined assuming the surface area was the same as at 421.4 m. For days when there was no record for lake stage, area and volume were assumed to be the same as for the previous recorded value for stage.

Volume of water directly entering the lake from atmospheric precipitation (rainfall, snow) was determined from daily precipitation values during 1980–1991 and from lake surface area on the days precipitation occurred. Values of daily evaporation for open-water periods were available from 1980 through 1991 from energy-budget and mass-transfer measurements. Volume evaporated for each day was determined by multiplying the daily evaporation rate by the same surface area of the lake used to determine energy-budget evaporation. During periods when the lake was ice covered, evaporation was considered to be zero; sublimation of ice was not considered for these budget calculations.

Groundwater input and lake discharge to groundwater were determined on a monthly basis. Hydraulic conductivity of adjacent aquifer material was determined by use of slug tests within each well as described by Bouwer and Rice (1976) and Bouwer (1989). The hydraulic gradient was determined from water table levels and lake stage. Segments of shoreline across which groundwater enters or leaves the lake were delineated on the basis of geologic deposits of the contiguous uplands and the presence of index wells considered to provide representative water-table gradients for that segment. Darcy's equation ($Q = KIA$, where Q is discharge, K is hydraulic conductivity, I is the hydraulic gradient, and A is the cross-sectional area of flow) was used to calculate groundwater fluxes. Heterogeneity in hydraulic conductivity was such that groundwater fluxes to and from the lake were calculated for eight sections of lakeshore. Three sections of the lakeshore were used to determine total input of groundwater (Fig. 1). Five sections of lakeshore were used to determine total loss of lake water to groundwater.

Calculation of chemical budgets

Chemical analyses of water collected by bulk and wet-deposition collectors at Williams Lake included determinations of silica, total and ortho-phosphorus, and Kjeldahl nitrogen. Mass of these constituents was calculated by multiplying the sum of daily precipitation values, for a period of time bracketing the date of sample collection, by the concentration of the constituents.

Measurement of major ions in precipitation at Williams Lake was not done during much of the study. However, chemical data for precipitation were available for the entire period of record from the Marcell Experimental Forest collection site of the National Atmospheric Deposition

Fig. 2. Comparison of monthly water inputs to Williams Lake from precipitation and groundwater.

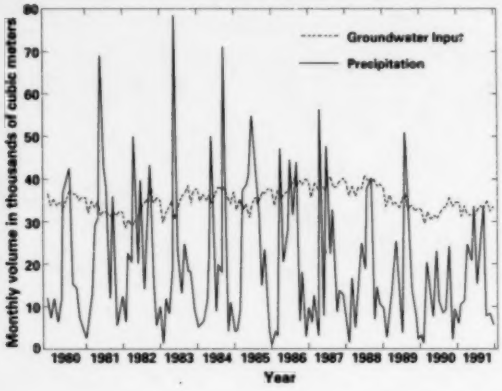
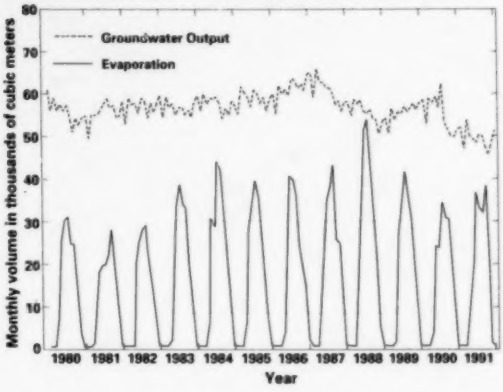


Fig. 3. Comparison of monthly water losses from Williams Lake by evaporation and seepage to groundwater.



Program/National Trends Network (National Atmospheric Deposition Program (NRSP-3)/National Trends Network, February 9, 1993, NADP/NTN Coordination Office, Natural Resource Ecology Laboratory, Colorado State University, Fort Collins, CO 80523, personal communication). This collection site is located approximately 100 km northeast of Williams Lake. Volume-weighted means for each month of record from the Marcell site were used to determine input of calcium, magnesium, sodium, potassium, chloride, sulfate, hydrogen ion, and dissolved inorganic nitrogen. Mass of these constituents entering Williams Lake through atmospheric precipitation was determined as the product of the volume-weighted mean concentration of the month and the volume of precipitation during the month. For the few times when concentration data were not available for a given chemical constituent, values from the preceding month were used in the calculation.

Mass of chemical constituents contributed to the lake from groundwater was determined from chemical analyses of water from three wells, each located in one of the areas used to calculate groundwater seepage to the lake. These three wells were selected for use in calculation of fluxes because they were the closest wells to the lake in the three areas of seepage to the lake and had been sampled often enough to enable the determination of temporal changes in chemical input. Days on which water was collected for chemical analysis were used to define midpoints of the periods of chemical input to the lake from groundwater. Scheider et al. (1979) found this method to result in the least uncertainty for calculation of flux. Concentrations from each of the three wells were multiplied by discharge from their respective areas of flow into the lake to calculate mass.

Concentrations of chemicals leaving the lake by discharge to groundwater were assumed to be the same as in the samples collected at 1 m below lake surface for major ions and silica and the samples collected from the photic zone for phosphorus and nitrogen. Day of sample collection defined the midpoint of the hydrological record used to calculate chemical mass leaving the lake. Concentration of each chemical constituent was multiplied by total lake discharge to groundwater to determine the mass leaving the lake.

Chemical mass within Williams Lake was calculated using concentration data from discrete depth and photic-zone samples. Chemical data from the sample collected at 1 m above lake bottom were multiplied by the volume of water below the 7.5-m depth of the lake. Specific conductance profiles indicated that when the lake was thermally stratified values below 7.5 m were not the same as the relatively uniform water column above 7.5 m; the sample collected at 1 m above lake bottom was below 7.5 m. Chemical data from the sample collected at 1 m below lake surface were multiplied by the difference between total lake volume on the day of sample collection and the volume below the 7.5-m depth of the lake. Mass from these two parts of the lake were added to obtain total mass in the lake. Chemical data from the photic zone were multiplied by the entire lake volume to determine mass. Change in mass was determined by difference between mass calculated for each day of sample collection.

Results

Water budgets

Groundwater movement into Williams Lake and lake discharge to groundwater were the largest hydrological fluxes in the water balance of the lake. Although the annual contribution of water to the lake from precipitation was less than 50% of total water input (Table 1), the contribution in some months was equal to, or exceeded, the contribution from groundwater (Fig. 2). Lake discharge to groundwater accounted for the greatest water loss from the lake on both a monthly (Fig. 3) and annual basis (Table 1). Loss of lake water by evaporation did not exceed loss to groundwater. The only time the two losses were about equal was in the summer of 1988.

The values for change in lake volume presented in Table 1 were determined from the actual changes in lake stage and the stage–volume relation, not by differences between measured water inputs and losses. In the ideal case where all components are measured exactly, the measured

Fig. 4. Monthly difference between measured water input and output and measured change in lake volume as a percentage of average lake volume.

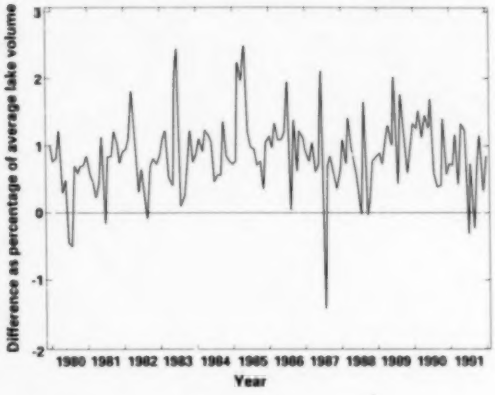
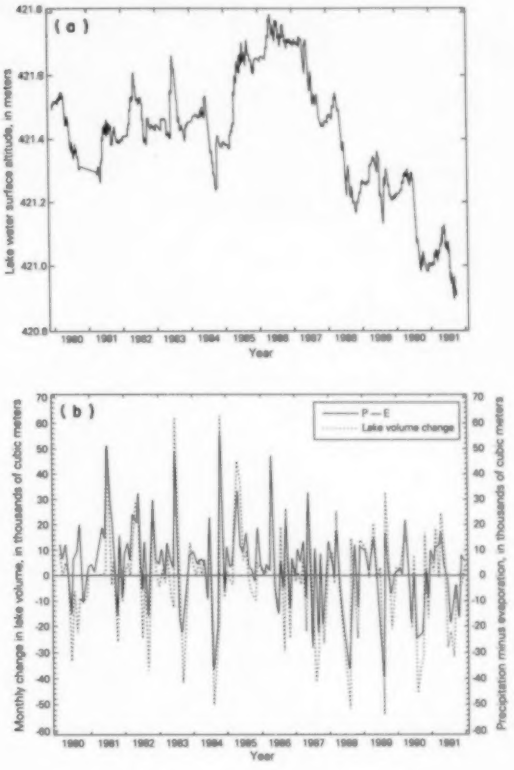


Fig. 5. (*a*) Daily change in altitude of lake surface. (*b*) Monthly change in lake volume and difference between precipitation and evaporation.



value for change in storage would be equal to the difference between measured inputs and outputs. The measured values for changes in storage in Williams Lake were not identical to the differences between water input and output (Fig. 4). Values in Fig. 4 usually were greater than zero because the monthly difference beween measured inputs and outputs was often negative and the measured change in storage was either a positive value or a negative value smaller than the difference between inputs and outputs. This suggested that either input was systematically underestimated or output was overestimated. However, this discrepancy between measured change in storage and the change in storage expected from the difference between measured inputs and outputs is related to the uncertainties in measurement of each component of the lake's water balance (Table 1). The fact that the discrepancy between measured components of the water balance was only 1% of average lake volume on a monthly basis indicates that the values presented here represent the hydrological processes controlling the water balance of Williams Lake.

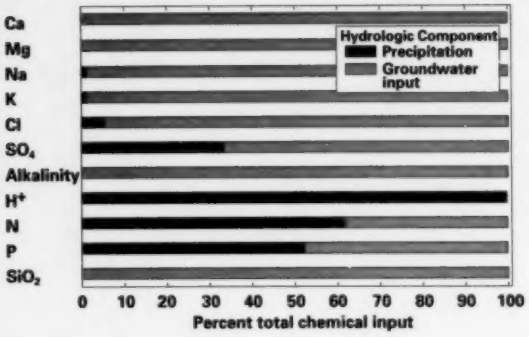
Stable isotope data also attest to the representativeness of the water budgets. In the period from mid-April 1991 to September 1991, water samples collected from bulk precipitation, the lake, and wells for analysis of $\delta^{18}O$ were used to calculate a mass balance for $\delta^{18}O$ following the method described by Krabbenhoft et al. (1990). In this method, when all of the isotopic values for all water-budget components are known, the volume of water contributed by any single unknown component can be determined as the difference between the other components' water volumes and isotopic values and the isotopic value of the unknown component. The estimate of the evaporative effect on $\delta^{18}O$ published by Krabbenhoft et al. (1990) was used in the calculation for Williams Lake. The water fluxes for April to September 1991 required for a $\delta^{18}O$ mass balance (groundwater input = 167 000 m^3, groundwater output = 224 000 m^3) compare well with independently estimated values (groundwater input = 172 000 m^3, groundwater output = 272 000 m^3). Hence our water-budget data seem to reflect the hydrological condition of Williams Lake.

Water renewal time, or residence time, commonly is determined by comparing lake volume to lake discharge. Average lake volume during 1980–1991 was 2.03 million m^3. Water residence time, based on annual values of lake discharge to groundwater, varied between 2.7 (1987) and 3.4 (1991) years. Thus, during the 12 years of this study, the average volume of the lake was removed at least four times by discharge to groundwater.

Differences between gains and losses of water resulted in changes of lake level over time (Fig. 5). Pronounced delines in lake level, occurring over periods of days to weeks, typically in late summer to autumn, coincided with periods of similar duration in which evaporation was large relative to precipitation. In 7 years of the study (1980, 1984, and 1987–1991) there was a net annual decline in storage of water in the lake. Six of these were years in which the annual difference between precipitation and evaporation was either a relatively small positive value (1984, 1987, 1991) or negative (1988–1990).

Pronounced short-term increases in lake level occurred in periods of peak precipitation when precipitation was large relative to evaporation (Fig. 5). The 5 years of the study (1981–1983, 1985, and 1986) in which there was net annual increase in storage had the largest annual difference between precipitation and evaporation. Although the

Fig. 6. Summary of the relative contribution of atmospheric deposition and groundwater to total chemical input.

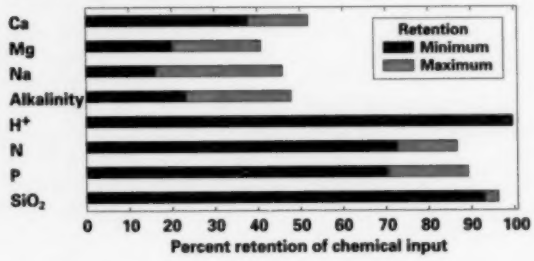


differences between precipitation and evaporation were related to lake level change, groundwater fluxes also had an effect. For example, those years with the largest positive differences between precipitation and evaporation, 1981 and 1982, did not have the largest increase in altitude of the lake water level.

Based on the residence time of water in the lake of approximately 3 years, periods in which relatively wet or relatively dry conditions persisted for more than a year would be expected to result in larger changes in lake level than observed in any one year. The nearly 1-m change in lake level from the peak in 1986 to the nadir in 1991 represented a change in lake volume of 14%, a change resulting from drier conditions in the last 5 years of the study. The largest increase in lake volume took place from mid-1984 to mid-1986 when there was a change in lake level of approximately 0.5 m. Thus, natural variability in climate over the 12-year period of study resulted in periods of quite different changes in lake level.

Although the 1984–1986 increase and 1982–1991 decline are extreme events for the period of study, these need to be placed in context with known variations in climate and the lake. The Palmer drought-severity index is an indication of periods of above- or below-normal moisture available in the soil for crop production (Palmer 1965). It is useful for determining the severity and duration of wet or dry meteorological conditions relative to normal conditions over periods of months to years. Palmer drought-severity index values for the vicinity of Williams Lake indicate 1987 to 1991 was similar to years of Midwestern drought in the 1930s; 1985–1986 was as wet as the wettest periods of the past century. Marl deposits in the watershed of the lake indicate the lake water level was about 6 m above modern levels at some undetermined time after the retreat of the continental ice sheet approximately 10 000 years before present (Seaburg 1985), likely as a result of ice damming in the watershed of the Shingobee River, which includes Williams Lake, although at present there is no surface-water connection with the river. Sediment cores from the lake indicate the lake water level was 4 m below modern levels during 7000 to 5000 years before present (S. Locke, Limnological Research Center, University of Minnesota, Minneapolis, personal communication). Thus, between 1987 and 1991, lake level in Williams Lake declined one quarter of the way towards the lower extreme of the past 10 000 years.

Fig. 7. Summary of the range of values for retention of chemical input for elements consistently retained in each year of the study.



Chemical budgets

Groundwater seepage into Williams Lake contributed nearly all of the annual input of calcium, magnesium, sodium, potassium, alkalinity, and silica (Fig. 6). Quartz, dolomite, calcite, and potassium feldspars are present in the glacial drift in the vicinity of Williams Lake (LaBaugh et al. 1981), and they are the primary source of these chemicals that enter the lake. Some of the annual input of chloride (~5%) and sulfate (~30%) entered the lake through atmospheric deposition (Fig. 6). Salt applied to roads in the region during the winter may be the source for chloride in atmospheric deposition. Sulfate measured in atmospheric deposition probably originates from a large region of sulfate-rich glacial drift to the west of the study area in North Dakota. Considerable airborne transport of fine particulates from this drift can occur as a result of agricultural activity and wind erosion from dry wetland and lake sediments. Only a small quantity of the annual input of hydrogen ions entered the lake through groundwater seepage to the lake (Fig. 6). Groundwater had circumneutral pH, whereas the pH of atmospheric deposition commonly was less than 6. Therefore, the concentration of hydrogen ions in groundwater was much smaller (less than one tenth) than the concentration in atmospheric deposition.

The amount of phosphorus supplied to the lake each year by groundwater was similar to the amount supplied by atmospheric deposition (Fig. 6). This determination was based on concentrations of soluble-reactive or orthophosphorus in water samples from the wells and total phosphorus concentration of atmospheric deposition. Concentrations of phosphorus in the wells were similar to that measured in groundwater flowing into the lake through sediment directly adjacent to shore. Several residences along the shoreline of the lake were occupied all year, but septic systems do not appear to be a source of phosphorus to the lake. Ortho-phosphorus concentrations in groundwater were similar to values found in wet deposition at the site. Similar to phosphorus, atmospheric deposition and groundwater contributed about equal amounts of dissolved inorganic nitrogen. Input of dissolved inorganic nitrogen was approximately one half of all nitrogen input from atmospheric deposition. The contribution of all forms of nitrogen by atmospheric deposition was usually more than 60% of input.

Neither the amount of chemical constituents supplied to the lake by groundwater and atmospheric deposition nor the amount lost from the lake by seepage was constant (Table 2). The relation between median values for each budget component in Table 2 are representative of the general relation for the 12-year study. Some constituents (calcium, magnesium, sodium, alkalinity, hydrogen, nitrogen, phosphorus, and silica) consistently had larger annual values of input than loss by seepage. There was retention of these constituents in the lake (Fig. 7). Median values of input of potassium and chloride were nearly equal to their respective values for output, which indicated little retention of input in the lake for these constituents. However, the difference between input and output for potassium and chloride was not consistent in sign (positive or negative value) or absolute amount in individual years. In the case of sulfate, sign and absolute amount of retention varied each year; the median value indicated some sulfate input was retained in the lake.

Retention of the constituents in the lake, presented in Fig. 7, indicated those constituents were not conservative. Thus, there may be some process that caused some of the input of these chemicals to remain in the lake. At the concentrations found in the lake water, both magnesium and sodium were expected to be conservative (Table 3). A possible reason for the mismatch between input and output could be related to the uncertainty in hydrological fluxes (Winter 1981), which propagates into uncertainty in chemical fluxes (LaBaugh 1985). The calcium to magnesium ratio in wells several meters downgradient from the lake is larger (~5) than in the lake (~2), which is similar (~2) to that in wells upgradient from the lake. These ratio data indicate that some magnesium is removed in outflow areas, possibly through some chemical process in the sediments as water seeps from the lake. The difference in the calcium:magnesium ratio in groundwater in areas of seepage out of the lake could also reflect spatial variability in geologic materials. Weathering of marls in the areas of outflow at the lake margin, which would have less magnesium relative to calcium than dolomite, would result in groundwater having a larger calcium:magnesium ratio than in areas where weathering of dolomite occurred. The possible role of sediments in the retention of magnesium may also help explain the apparent retention of sodium as well, although, as stated above, this apparent retention may be due to uncertainty in the hydrological fluxes.

The change in mass of calcium in the lake had a definite seasonal pattern each year (Fig. 8). During the period of declining mass of calcium, from May to August, water in the lake commonly was supersaturated with respect to calcite. Removal of dissolved CO_2 from lake water by photosynthesis caused precipitation of calcite, which removed calcium from lake water. Calcite was found in sediment cores taken from the lake bottom (LaBaugh et al. 1981). The difference between the amount of calcium supplied annually to the lake by groundwater and atmospheric precipitation and the amount lost from the lake by lake discharge to groundwater was approximately the same as the independently determined seasonal change in mass. Loss of calcium by biologically mediated precipitation of calcite is a mechanism that could account for the fact that hydrological transport

Table 2. Chemical-budget data for Williams Lake, 1980–1991. All chemical-flux data are in thousands of moles per year except for alkalinity, which is in thousands of equivalents per year, and hydrogen and phosphorus, which are in moles per year.

Budget component	Calcium	Magnesium	Sodium	Potassium	Chloride	Sulfate	Alkalinity	Silica	Nitrogen	Phosphorus	Hydrogen Ion
Inputs											
Atmospheric deposition	**1.28**[a] *0.58–1.85*[b]	**0.35** *0.17–0.72*	**0.56** *0.25–2.50*	**0.21** *0.10–0.31*	**0.53** *0.31–0.98*	**2.97** *1.34–3.98*	**0.31** *0.01–9.51*	**0.308** *0.020–0.436*	**13.5** *8.7–20.2*	**82** *26–325*	**2.22** *1.12–3.05*
Groundwater input	**724** *654–837*	**292** *256–364*	**58** *45–68*	**17** *16–21*	**13** *8–56*	**6.4** *4–23*	**2004** *1613–2281*	**126** *106–136*	**11.1** *9.0–15.5*	**66** *59–114*	**0.13** *0.008–0.02*
Outputs											
Lake seepage to groundwater	**405** *370–424*	**211** *191–219*	**41** *37–43*	**18** *15–23*	**12** *9–20*	**9.7** *0–22*	**1251** *1139–1307*	**6.8** *4.8–7.4*	**5.1** *3.8–8.3*	**27** *17–82*	**0.007** *0.004–0.016*
Inputs minus outputs	**320** *252–433*	**87** *55–150*	**17** *8–33*	**1.6** *(−5.7)*[c] *– 3.5*	**2.6** *(−5) – 46*	**5.2** *(−13) – 15*	**737** *376–1052*	**120** *99–130*	**18.5** *15–27*	**117** *8–370*	**2.22** *1.13–3.05*
Change of mass in lake water	**3.5** *(−138) – 119*	**(8.5)** *(−49) – 63*	**0.8** *(−18) – 15*	**(−1.7)** *(−26) – 33*	**4.7** *(−24) – 75*	**4.7** *(−157) – 132*	**(−12)** *(−168) – 359*	**(−0.9)** *(−13.7) – 22.8*	**2** *(−267) – 214*	**(−36)** *(−4934) – 5111*	**0.006** *(−0.046) – 0.024*

[a]All values in **bold** are median annual values for the 12-year period of study.
[b]All values in *italics* represent the range in yearly values.
[c]All values in parentheses are negative numbers.

Table 3. Median chemical concentration data for Williams Lake, adjacent groundwater, and precipitation collectors at the study site and data from the Marcell Experimental Forest. Units are millimoles per liter, except for alkalinity, which is in milliequivalents per liter, and pH.

Water source	Calcium	Magnesium	Sodium	Potassium	Chloride	Sulfate	Alkalinity	pH
Williams Lake (1 m below surface)	0.573	0.300	0.056	0.025	0.016	0.012	1.778	8.1
Williams Lake (1 m above bottom)	0.623	0.304	0.056	0.025	0.016	0.012	1.918	7.7
Groundwater (in area of seepage to lake)	1.621	0.616	0.102	0.028	0.059	0.084	4.456	7.6
Groundwater (in area of seepage from lake)	1.94	0.452	0.108	0.040	0.033	0.011	4.996	7.3
Precipitation (wet from Marcell)*	0.0052	0.0016	0.0024	0.0007	0.0025	0.0118	—	5.0

*Data from Marcell Experimental Forest collection site of the National Atmospheric Deposition Program/National Trends Network.

Fig. 8. Change in mass within the lake for calcium and alkalinity showing seasonal changes over the period of study.

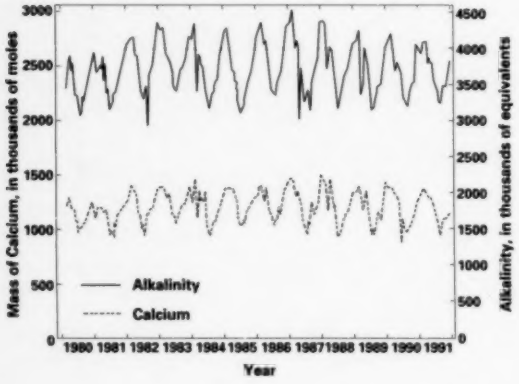
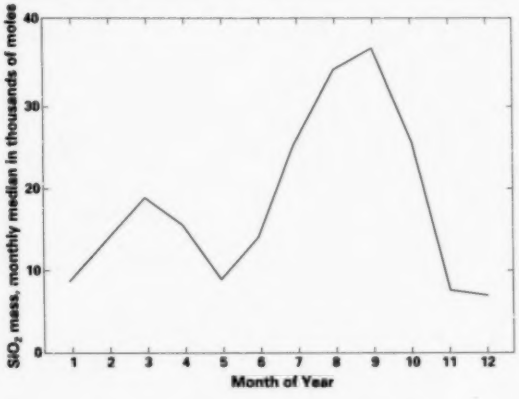

Mass of Calcium, in thousands of moles
Alkalinity, in thousands of equivalents
Alkalinity
Calcium
1980 1981 1982 1983 1984 1985 1986 1987 1988 1989 1990 1991
Year

Fig. 9. Seasonal change in the mass of silica. Values are medians for each month of the year.



of calcium out of the lake was less than input from groundwater and atmospheric deposition.

Alkalinity in the lake changed seasonally in a similar pattern to calcium (Fig. 8), which was consistent with loss of both owing to precipitation of calcite. Alkalinity supplied to the lake by hydrological inputs was retained, but not by a percentage similar to that of calcium (Fig. 7). Estimates of dissolved inorganic carbon represented by alkalinity were made using the geochemical model PHREEQE (Parkhurst et al. 1990). Groundwater input to the lake also supplied most of the annual input of dissolved inorganic carbon from hydrological fluxes. Between 30 and 50% of the input from groundwater was retained. The absolute amount of dissolved inorganic carbon retained in the lake each year was larger than the measured change in storage. Based on fluxes of dissolved inorganic carbon associated with hydrological fluxes, and assuming all of the retained dissolved inorganic carbon was used in photosynthesis, between 0.3 and 0.5 mol $C \cdot m^3$ (1.5–2.8 mol $C \cdot m^2$) were removed from the water column of the lake each year by photosynthesis. Measurement of gas exchange with the atmosphere was not part of this study; however, partial pressure values for CO_2 in midsummer, calculated using PHREEQE (Parkhurst et al. 1990), indicated depletion below atmospheric equilibrium values, indicating an influx of CO_2. This additional input of inorganic carbon indicates that the removal of dissolved inorganic carbon from the water column by biota could represent larger values than estimated from differences in hydrological fluxes alone.

Annual silica input was nearly all retained in the lake. The difference between change in mass in the lake and the surplus of input of silica on an annual basis was between 1.6 and 3.9 times the maximum mass present in the lake each year. Mass of silica in the lake changed seasonally, declining in spring from a small late-winter peak, and in fall following a large, late-summer peak (Fig. 9). The minima are coincident with increased abundance of diatoms and chrysophytes (J.W. LaBaugh, unpublished data). Only in summer months did the monthly change in mass in the lake equal or exceed that expected from the difference between inputs and outputs (Fig. 10). Thus, utilization of silica by biota is the likely mechanism for removal of silica from water in the lake. This biologically mediated loss of silica from the water column represented an annual removal rate of 12–21 $g \cdot m^{-2}$ of lake surface.

Fig. 10. Monthly change in mass of silica in the lake compared with measured difference between input and output owing to hydrological fluxes.

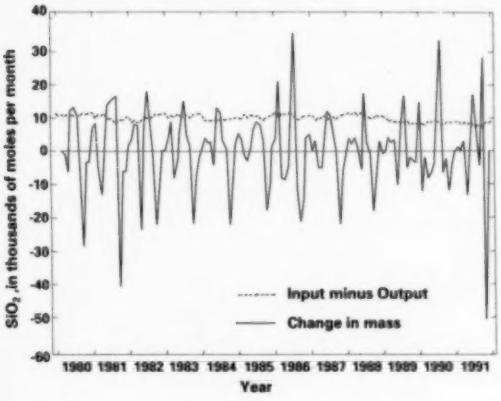
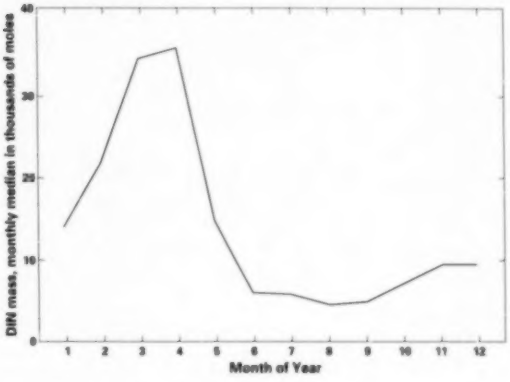


Fig. 11. Seasonal change in the mass of dissolved inorganic nitrogen (DIN). Values are medians for each month of the year.



Within the lake, pH of water in the epilimnion changed seasonally, and values of pH usually exceeded 8.5 in warmer months, reflecting loss of hydrogen from solution by the shift in the bicarbonate equilibrium caused by photosynthetic use of dissolved carbon dioxide in lake water. More than 99 percent of annual input for hydrogen ion remained in the lake (Fig. 7).

Concentrations of ortho-phosphorus in the lake were used to determine loss of phosphorus from the lake through lake seepage to groundwater. Thus, dissolved inorganic phosphorus was the only form considered to move into or out of the lake through the lake sediments and adjacent geological material. The lake retained greater than 70% of annual phosphorus input. The difference between input and output of phosphorus always showed a surplus of input (Fig. 7). Change in mass of phosphorus in the lake varied. For some years the increase in mass was greater than that expected by difference between input and output; for other years, the mass decreased. These results indicated sedimentation, and resuspension of sediments probably had a greater role in controlling mass in the lake than the difference between hydrologically controlled fluxes. The difference between input and output usually represented less than 10% of the maximum mass in the lake.

Mass of dissolved inorganic nitrogen in the lake changed seasonally, increasing under ice cover to a maximum in April, then declining to minimal values from June to September (Fig. 11). These seasonal changes were larger than expected, based on the difference between input and output. More than 60% of the annual input of dissolved inorganic nitrogen was retained in the lake. Seasonal changes likely reflect uptake by biota and mineralization. However, on an annual basis the surplus of dissolved inorganic nitrogen flux to the lake was larger than the annual change in dissolved inorganic nitrogen in the lake, indicating additional loss of nitrogen from the lake other than lake discharge to groundwater. Changes in total nitrogen content of the lake were not consistent with those expected from the surplus of total nitrogen from atmospheric deposition and groundwater input (70%). Changes in mass of total nitrogen varied from year to year, often indicating a net loss.

The annual chemical input from either atmospheric deposition or groundwater and the chemical output to groundwater were not constant (Table 2). This was not surprising given the natural variability in water flux represented by inputs and outputs (Table 1). Because of the extremes in hydrological conditions during the 12-year period of study, extremes consistent with extremes in the past century, it is useful to examine what the effect of these extremes were on the chemical characteristics of the lake. Although annual precipitation input of water declined after the peak year 1985, the largest annual input of water from groundwater occurred in the following 3 years (Table 1), resulting in the largest annual input of calcium from groundwater in those same 3 years (Fig. 12). These data indicate that the effect of the wetter period of 1985–1986, in terms of precipitation, persisted in the form of increased water and chemical input from groundwater for a few years past the peak in precipitation input to the watershed of the lake.

Despite the larger input of calcium in 1986–1988, peak values for mass of calcium in the lake declined after 1986 (Fig. 8). At this time output of calcium by lake seepage out of the lake did not increase to the same extent that input did. Thus, larger values might have been expected for mass in the lake than were measured. Clearly, annual differences in groundwater input alone are not sufficient to explain why mass of calcium in the lake began to decline while input increased without much change in loss to groundwater. In 1987 and subsequent years the amount of phosphorus input to the lake was larger than in previous years (Fig. 13). Increased photosynthesis in response to this increase in phosphorus input may have resulted in the largest decline in calcium content in the open-water period during the study, which occurred in 1987 (Fig. 8). Decreased input of calcium in the drier years of 1989–1991 also contributed to the declining peak values of calcium in the lake during that period. These data indicate that, to understand the effect of extremes in climate such as occurred in the 12-year period reported here, the interaction of

Fig. 12. Comparison of annual input and output of calcium.

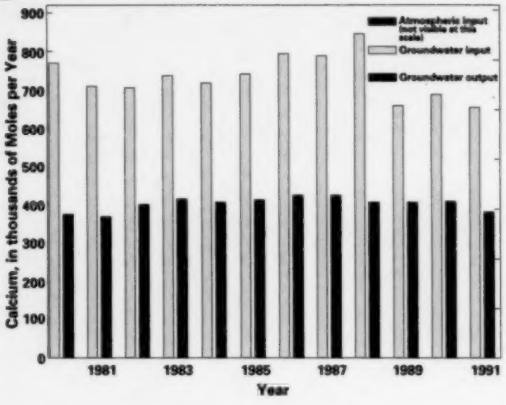
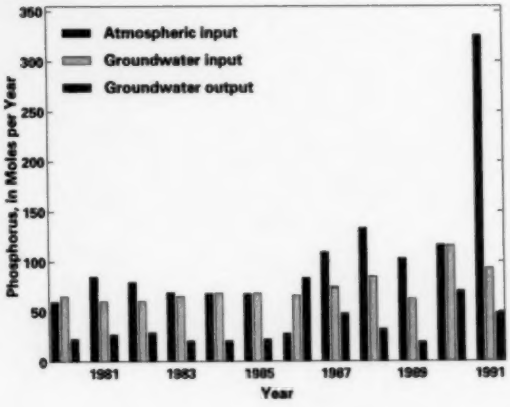


Fig. 13. Comparison of annual input and output of phosphorus.



groundwater input, groundwater output, precipitation input, and in-lake processes need to be understood. Also, antecedent conditions play a role; the wet period between 1985 and 1986 ameliorated the effect of decreased input of water from precipitation in subsequent years.

Discussion

Several investigators have pointed out the usefulness of using chemical budgets to evaluate ecosystem processes in aquatic and terrestrial environments (Schindler et al. 1976; Gorham et al. 1979; Likens 1985). Calcium retention in Williams Lake points to the importance of an in-lake removal mechanism, biologically mediated precipitation of calcite due to photosynthesis by algae and submerged aquatic plants. Seasonal changes in the mass of calcium within the lake were similar to the surplus of input. Otsuki and Wetzel (1974) also found precipitation of calcite to be an important removal process for calcium in Lawrence Lake, Michigan. Calcium can also be supplied by weathering of silicates, as shown by Schindler et al. (1976), Likens (1985), Jeffries et al. (1988), and Kenoyer and Bowser (1992). However, in these more dilute systems calcium was not retained. In the case of the Turkey Lakes watershed in central Ontario, an export surplus of calcium associated with alkalinity generation and calcium exchange occurred in response to input of sulfate (Jeffries et al. 1988). Turner et al. (1990) provide a summary of the mechanisms involved in alkalinity generation in lakes affected by atmospheric sulfate deposition.

Chemical elements that are considered to be conservative have been used in budget analyses to determine if estimates of hydrological fluxes are reasonable, particularly if a budget component is estimated by difference (Krabbenhoft et al. 1990). Even when stable isotopes are used in such an analysis (Krabbenhoft et al. 1990), uncertainty in the value for evaporation can cause problems (Gat and Gonfiatini 1981; Stauffer 1992; Krabbenhoft et al. 1992). In freshwater systems, magnesium has been used to evaluate assumptions as to how well hydrologic fluxes have been defined (Otsuki and Wetzel 1974; Stauffer 1985). Hydrological and stable-isotope data for Williams Lake indicated the water fluxes used to calculate chemical budgets were reasonable. However, even though magnesium commonly is considered to be conservative, it was apparently retained by the lake. Frape and Patterson (1981) indicated that interaction of groundwater with sediment, as water moved into Perch Lake, Ontario, affected the chemical characteristics of the water that eventually entered the lake. Data presented here for Williams Lake indicate that sediments also may affect the chemical characteristics of water seeping out of the lake. It also is possible that the perceived retention of magnesium is within the limits of uncertainty in the budget. For example, LaBaugh (1985) indicated uncertainty in the calculation of the retention of a chemical is a function of uncertainty in measurement of water budget.

Among the few studies of chemical fluxes to and from lakes that included direct measurement of groundwater, some indicated that groundwater contributions were negligible (Malueg et al. 1975; Schindler et al. 1976). However, many hydrological investigations have documented a wide range in the contribution of groundwater to lakes. For example, Shaw and Prepas (1990) reported that, for nine lakes in various geologic settings in Alberta, the groundwater contribution to the lakes varied from 4 to 49% of all fluxes to the lakes. Groundwater seepage into several lakes in Wisconsin, including Sparking Lake, varied between ~2 and ~30% as summarized by Baker et al. (1990). At Beaver Lake, Wisconsin, Brown and Cherkauer (1991) reported that groundwater comprised 70% of all fluxes to the lake and 60% of all fluxes from the lake. Data from Williams Lake indicate that more than 70% of the sum of all hydrological fluxes to and from the lake were attributed to groundwater. At Sparking Lake, Wisconsin, noted above, Krabbenhoft et al. (1990) found that groundwater inputs were nearly equal to atmospheric precipitation and that output to groundwater was about one half the loss by evaporation. Williams Lake and Sparking Lake are underlain by unconsolidated glacial outwash, indicating groundwater contributions to lakes can be significant when lakes are in such geologic settings.

Geologic settings also influence the major ion contributions from groundwater to lakes. In the case of Williams

Lake, the calcareous drift weathers to produce large concentrations of calcium, magnesium, and bicarbonate alkalinity in groundwater discharging to the lake. Combined with losses of lake water by seepage to groundwater, and biologically mediated precipitation of calcite, groundwater chemistry controls the chemical composition of the lake. Calcareous drift also affected the groundwater composition near Lawrence Lake, Michigan, where groundwater contributed as much as 58% of the monthly input of calcium (Otsuki and Wetzel 1974). Even in noncalcareous, coarse-grained material, Kenoyer and Anderson (1989) found groundwater to be a significant source of major ion input, although groundwater contributed only 10% of water input to Crystal Lake, Wisconsin. In finer grained material, the volume of water contributed by groundwater may be small relative to other sources, but groundwater can contribute much of the major ion input (Schwartz and Gallup 1978).

Similarly to what Hurley et al. (1985) found for Crystal Lake, Wisconsin, a surplus of silica input from groundwater was found in Williams Lake. The large value for retention of silica for Williams Lake could be a result of use by biota. Schindler et al. (1976) also indicated Rawson Lake retained approximately 40% of its silica input owing to use by biota, and Likens (1985) found Mirror Lake retained 56% of its silica input. Input of silica to Rawson Lake and Mirror Lake was primarily from streams.

Other studies of groundwater fluxes to lakes have indicated groundwater can be a source of phosphorus. Furthermore, much of this input can result as groundwater moves through littoral sediments containing pore water concentrations that are larger than those in groundwater (Brock et al. 1982; Shaw et al. 1990). Concentrations of phosphorus in the shallow sediments where groundwater flows into Williams Lake were similar to concentrations in nearby wells. Yet, groundwater contributed almost as much input as atmospheric deposition. Retention of phosphorus in the lake, between 70 and 90%, is similar to that measured for Rawson Lake, Ontario (67–79%) by Schindler et al. (1976) and Clear Lake, Ontario (73%), by Schindler and Nighswander (1970).

Retention of nitrogen in Williams Lake was similar to that for phosphorus; between 70 and 90% of nitrogen input from hydrologic fluxes was retained. This retention is consistent with use by biota in the lake, as has been found in other lake studies where nitrogen retention has been 72–84% (Schindler et al. 1976), 59% (Likens 1985), or 98% (Schindler and Nighswander 1970). Hydrologic processes are not the only fluxes affecting the nitrogen balance of aquatic systems; exchange with the atmosphere also can play a role. However, atmospheric exchange is rarely quantified in conjunction with studies that also include direct determination of hydrological fluxes (Schindler et al. 1976). The few studies that have documented groundwater input of nitrogen found it to be negligible (Malueg et al. 1975; Schindler et al. 1976), or at most, 2% of hydrologically controlled input (Brook et al. 1982). This study of Williams Lake indicates it is possible to have almost as much input of nitrogen by groundwater as from atmospheric deposition.

In the few studies that have directly measured groundwater contribution to the water and chemical fluxes for a lake, budgets have rarely been determined for more than a single year. Schindler et al. (1976) noted that chemical fluxes for watersheds vary sufficiently from year to year to necessitate several years of data for accurate chemical budgets. The length of the study of Williams Lake provided the opportunity to examine the variability of fluxes. Year-to-year variation in inputs of major cations, sulfate, and alkalinity from atmospheric deposition was greater than for groundwater input. Inputs of calcium, magnesium, sodium, and alkalinity from groundwater were consistently smaller during the last 3 years of the study, the driest years of the investigation.

Anderson and Cheng (1993) presented in-lake concentration data to infer that changes resulting from short- and long-term changes in groundwater flow to Crystal Lake, Wisconsin, could have an effect on the chemical budgets of the lake. For Crystal Lake, decreased groundwater input was expected to result in declines in pH and silica in the lake. The directly measured water and chemical budgets for Williams Lake indicated that month-to-month and year-to-year variation in chemical fluxes could be associated with changes in atmospheric deposition as well as groundwater. Some of these changes were large enough to affect mass in the lake within the time in which the entire volume of the lake and mass in the lake were replaced. Declining inputs of alkalinity in the last 3 years of this study of Williams Lake coincided with decreased mass in the lake. Thus, response of groundwater flow systems to changing climate conditions can have an effect on chemical characteristics of the lake.

Conclusions

This study of Williams Lake indicated that interaction with groundwater determined the hydrological and chemical characteristics of the lake. Seasonal cycles in calcium, alkalinity, and silica indicated biological processes could cause removal of those chemical constituents, and budget data indicated those losses could be compensated by groundwater input. Data presented here indicate flux into the lake from groundwater may be an important driving mechanism for biological processes in lakes that have no surface-water input. Some remineralization of these elements after uptake by biota is likely. Lake seepage to groundwater also removed large fractions of the annual input of several chemical constituents. Examination of the water budget of the lake indicated a net loss of water, yet the chemical budgets clearly indicated net loss of water did not result in a net loss in chemical mass. Attempts to understand the role of groundwater in chemical fluxes of lakes, where groundwater flux is determined as a net value, may be misleading.

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Size-specific fish avoidance of the spined crustacean *Bythotrephes*: field support for laboratory predictions

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Abstract: Stomach contents of juvenile fish and zooplankton samples taken simultaneously with fish were analyzed to determine whether young fish in Lake Superior utilize *Bythotrephes* (*cederstroemi* form), a spined crustacean present in the Great Lakes since the 1980s. *Bythotrephes* made up approximately 11% of the zooplankton samples on our sampling date; however, it comprised approximately 0.3% of the fish diet (N = 358 fish, 2.8–23.9 cm in total length). No fish <7.0 cm in length (N = 208) contained *Bythotrephes*. According to the Manly–Chesson selectivity index, fish negatively selected or avoided the spined zooplankter. A Spearman rank correlation analysis indicated that although the number of *Bythotrephes* in the gut significantly increased with fish length, preference did not. These field results support predictions derived from laboratory experiments that (1) *Bythotrephes* is not a preferred prey item for young fish, (2) there is a threshold size of fish that can utilize *Bythotrephes* as a food source, and (3) as fish increase in size, they are more likely to consume *Bythotrephes*.

Résumé : Les contenus stomacaux de poissons juvéniles et des échantillons de zooplancton prélevés en même temps que les poissons ont été analysés afin de déterminer si les jeunes poissons du lac Supérieur se nourrissaient de *Bythotrephes* (forme *cederstroemi*), un crustacé épineux présent dans les Grands Lacs depuis les années 1980. Les *Bythotrephes* représentaient 11% environ des échantillons de zooplancton que nous avons prélevés, mais ils ne constituaient que 0,3% des aliments ingérés par les poissons (N = 358 poissons de 2,8 à 23,9 cm de longueur totale). Aucun poisson de moins de 7,0 cm de longueur (N = 208) contenait des *Bythotrephes*. Selon l'indice de sélectivité de Manly–Chesson, les poissons effectuaient une sélection négative, ou évitaient, l'organisme zooplanctonique à épine. Une analyse par corrélation de rangs de Spearman a montré que bien que le nombre de *Bythotrephes* présents dans l'appareil digestif augmentait avec la taille, il n'y avait pas d'accroissement de la préférence. Les résultats des travaux sur le terrain appuient les prévisions obtenues suite à des travaux en laboratoire et selon lesquelles: (1) les *Bythotrephes* ne constituent pas une proie recherchée par les jeunes poissons (2) il existe un seuil de taille chez les poissons pour la consommation des *Bythotrephes* et (3) la probabilité de consommation de ces organismes s'accroît avec la taille des poissons.
[Traduit par la Rédaction]

Introduction

The large, spined zooplankter *Bythotrephes cederstroemi* (Fig. 1) invaded the Great Lakes in the 1980s (Bur et al. 1986; Lange and Cap 1986; Lehman 1987; Evans 1988; Cullis and Johnson 1988) and invaded inland lakes in the United States and Canada in the 1990s (Yan et al. 1992). As a predator on other zooplankton (Mordukhai-Boltovskaia 1958) and an important prey for fish (e.g., Nilsson 1979), it likely occupies an intermediate position in the Great Lakes food web, sharing the zooplankton resource with other invertebrate predators and small fish, and for them, representing a food source. However, laboratory experiments by Barnhisel (1991*a*, 1991*b*) indicated that *Bythotrephes* is protected from small fish predators <10 cm in length due to a barbed caudal spine that comprises up to 80% of its length (Fig. 1). In feeding experiments with age-0 rainbow trout (*Oncorhynchus mykiss*), 4.0–8.0 cm in length, the presence of *Bythotrephes*' caudal spine increased foraging time 800%. Foraging time increased as fish size

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Fig. 1. Adult *Bythotrephes cederstroemi* approximately 1 cm in length. The spinelike appendage extends caudally with three pairs of lateral barbs. Each pair of barbs corresponds to a molt and can be used to identify instars. Drawing by N.L. Korda.

decreased (Barnhisel 1991*a*). Age-0 yellow perch (*Perca flavescens*), 5.0–6.0 cm in length, exhibited a variety of behaviors including aversion as they foraged on *Bythotrephes*. When the same perch were fed *Bythotrephes* with varying portions of the spine removed, aversive behaviors decreased as more of the spine was removed (Barnhisel 1991*b*). In laboratory experiments by Parrish and Margraf (1991) to determine prey preferences of age-0 yellow perch and white perch (*Morone americana*), both species negatively selected *Bythotrephes*.

From these studies, we predicted that (1) *Bythotrephes* would not be a preferred prey item for young or small fish due to its tail spine, (2) there may be a size threshold at which fish begin to utilize *Bythotrephes* as a food source, and (3) fish are more likely to consume *Bythotrephes* as they increase in size. We addressed these predictions with field data to clarify to what degree small fish are actually utilizing *Bythotrephes*, and consequently, to what degree *Bythotrephes* provides an alternative resource to small fish as it shares or competes for the zooplankton resource.

Materials and methods

We analyzed gut contents of juvenile fish, and plankton samples taken simultaneously with fish, from Keweenaw Bay, Lake Superior. Fish were collected by the National Biological Survey, Great Lakes Center (formally a part of the U.S. Fish and Wildlife Service), Ashland Biological Station, during routine seasonal trawling at their Bete Grise station (latitude 47°20′; longitude 87°50′) on 20 September 1991. A bottom trawl with an 11.9-m head rope, a 15.6-m foot rope, and a 12.7-mm-stretched-mesh cod end was used to collect fish from depths of 16–53 m. A total of eight bottom trawl tows were taken (Table 1). Age-0 and age-1 rainbow smelt (*Osmerus mordax*) were caught in the first six tows. Age-1 rainbow smelt, age-0 lake herring (*Coregonus artedi*), and age-0 to age-2 lake whitefish (*Coregonus clupeaformis*) were caught in remaining tows over greater depths. Fish were immediately frozen on ship, transported to our laboratory, and thawed.

We measured fish total length and girth to the nearest 0.1 mm and identified stomach contents to genus or functional group based on size (Table 1). Large, relatively rare zooplankters (e.g., *Bythotrephes* and *Mysis*) were counted directly. Smaller, more numerous zooplankters were categorized as either cladocerans or copepods and counted in subsamples. Depending on the size of fish and amount in the gut, stomach contents were diluted to 100 mL and three 1/10 volumes were counted. Fish with empty stomachs ($N = 9$; young-of-the-year (YOY) rainbow smelt) or stomach contents too badly decomposed ($N = 7$; four lake herring; three rainbow smelt) were excluded from the study.

Plankton samples were taken from one of three depths (20, 30, and 40 m) to the surface using a 0.35-mm-mesh net, 0.5 m wide and 2.0 m long, as trawl tows were conducted over increasingly deeper water. Three replicate samples were taken for each depth for a total of nine collections (Table 2). Surface temperature was approximately 14°C; temperatures at depths of 20, 30, and 40 m were approximately 11°C. Large, relatively rare zooplankters were counted directly. *Bythotrephes* was identified according to the number of barbs on the caudal appendage. We recorded the reproductive status of animals with three barbs (mature instar stage) and counted spines occurring separate from the body. To count smaller, more numerous zooplankters, the sample was reduced to 100 mL and three 10-mL subsamples were counted. Cladocerans and copepods were identified to genus. Copepods (*Limnocalanus*, *Senecella*, *Epischura*, diaptomids) were categorized as either large or small depending on size (i.e., large ≥2 mm; small <2 mm).

Fish preferences for prey were calculated using the Manly–Chesson selectivity index, alpha (Chesson 1983).

Table 1. Trawl, fish, and stomach content information. Fish from trawl tows 2 and 3 were divided into two groups based on size. The *Bythotrephes* category consists of animals with spines having two and three pairs of barbs and spines occurring alone. Copepods were functionally grouped according to size: large individuals (Lg) were ≥2 mm (mainly *Limnocalanus*, *Senecella*, *Epischura*) and small individuals (Sm) were <2 mm (mainly diaptomids). Rainbow smelt taken from <48 m contained small copepods.

Trawl		Fish				Stomach contents (mean No. per fish)				
			Total length (cm)					*Bythotrephes* (mean (SE))		
No.	Depth (m)	*N*	Range	Mean (SE)	Girth (cm) (mean (SE))	Copepoda (mean (SE))	Cladocera (mean (SE))	Animals	Spines	*Mysis* (Mean (SE))
Rainbow smelt (*N* = 243)										
1	16	30	3.0–4.0	3.5 (0.5)	0.4 (0.1)	20.4 (2.8)	0.6 (0.2)	0	0	0
2	22	30	2.8–4.6	3.8 (0.1)	0.5 (0.0)	24.4 (2.7)	2.0 (0.3)	0	0	0
		20	4.9–7.9	6.2 (0.2)	1.0 (0.0)	57.9 (6.8)	15.5 (2.0)	0	0	0
3	27	30	3.0–5.2	3.9 (0.0)	0.5 (0.0)	32.4 (3.7)	0.6 (0.2)	0	0	0
		5	5.5–7.7	6.6 (0.4)	1.0 (0.9)	30.4 (5.6)	10.2 (1.6)	0	0	0
4	31	30	3.2–5.4	4.0 (0.1)	0.5 (0.0)	24.0 (4.7)	1.4 (0.5)	0	0	0
5	35	34	2.9–4.3	3.5 (0.0)	0.5 (0.0)	6.1 (1.2)	0.1 (0.1)	0	0	0
6	40	30	3.2–4.3	3.8 (0.0)	0.5 (0.0)	9.1 (1.3)	0.6 (0.2)	0	0	0
7	48	34	7.9–14.0	10.2 (0.2)	1.8 (0.1)	Small: 114.1 (11.9)	375.0 (21.8)	0.5 (0.2)	0	0
						Large: 29.4 (6.7)				
Lake herring (*N* = 85)										
7	48	39	6.3–15.5	9.1 (0.3)	2.0 (0.1)	Small: 238.2 (28.7)	384.3 (38.5)	1.2 (0.4)	0.2 (0.1)	0
						Large: 60.2 (9.0)				
8	53	46	6.2–11.3	8.5 (0.2)	1.9 (0.1)	Small: 118.3 (12.2)	241.5 (17.6)	0.5 (0.2)	0.4 (0.1)	0
						Large: 44.6 (7.3)				
Lake whitefish (*N* = 30)										
8	53	30	8.3–23.9	12.3 (0.5)	2.7 (0.1)	0	0	1.5 (0.7)	1.6 (0.3)	20.5 (2.1)

Preference for prey i, alpha i, in the presence of m prey species is given by

$$i = \frac{\ln[(n_i - r_i)/n_i]}{\sum_{j=1}^{m} \ln[(n_j - r_j)/n_j]}$$

where r is the number of prey i consumed and n is the number of prey i available to the predator. Alpha values range from 0, indicating negative selectivity or avoidance, to 1, indicating positive selectivity or preference. Neutral selectivity is implied by an alpha value that is not significantly different from 0.5 and assumes that the predator took prey in proportion to their abundance in the environment. To test whether alphas differ significantly from 0.5, values were arcsine transformed to meet the assumption of normality (Sokal and Rohlf 1981). Transformed values were compared with the arcsine-transformed value of 0.5 using Student's t-test and a probability level of 0.01. To ensure that alpha estimates did not include prey unavailable to the predator, we calculated alpha only for those fish greater or equal in length to the smallest size at which *Bythotrephes* occurred in the gut. We did not calculate alpha for lake whitefish because *Mysis*, a dominant food item, did not appear to be adequately collected by plankton tows (Table 2). Alpha was calculated using four prey groups as r, prey consumed: cladocerans, large and small copepods, and *Bythotrephes* (all animals with two and three pairs of barbs and spines occurring alone). *Bythotrephes*' spines are large, robust structures that are often used as indicators of *Bythotrephes* consumption (Evans 1988; Mills et al. 1992). We used the average number of prey captured from the 0–40 m plankton tows to estimate n, prey available (Table 2).

We considered the size of the smallest fish having a *Bythotrephes* in its gut to indicate the size at which fish begin to consume the zooplankter. To determine whether fish are more likely to consume *Bythotrephes* as they increased in size, we correlated the number of prey found in the gut with fish length and calculated the Spearman rank correlation coefficient, r_S (Sokal and Rohlf 1981). A correlation analysis was conducted for fish whose length was greater than or equal to the species size threshold for *Bythotrephes* consumption regardless of whether *Bythotrephes* occurred in the gut. This assumed that fish greater than or equal to the size threshold were able to consume *Bythotrephes* even if they did not and that spines represented a recently eaten, digested *Bythotrephes*. Spines and zero values were excluded and the data reanalyzed to determine how sensitive the analysis was to our assumptions. We included other prey species for comparison. To determine whether preference increased with length, we correlated transformed alpha values with fish length.

Results

Of the 358 fish analyzed, *Bythotrephes* comprised approximately 0.3% of the diet. It occurred only in fish >7.0 cm caught from depths ≥48 m (Table 1). In contrast, *Bythotrephes* (excluding neonates and spines) made up approximately 11% of the zooplankton (as collected by our plankton tows) and was present throughout the water column (Table 2) in abundances of approximately 48 individuals·m^{-3}.

The number of *Bythotrephes* found in fish stomachs according to fish length and species is presented in Fig. 2. Rainbow smelt taken at depths of 16–40 m that contained no *Bythotrephes* (N = 209; 2.8–7.9 cm; Table 1) were not included in the figure. The smallest fishes that contained a *Bythotrephes* were a 10.2-cm rainbow smelt, a 7.3-cm lake herring, and a 9.4-cm lake whitefish. For age-1 rainbow smelt from 48 m, 76% had not consumed a *Bythotrephes*, while 75% of lake herring had not consumed a *Bythotrephes*. For lake whitefish, 36% contained intact *Bythotrephes* whereas 47% contained only the spine. Spines comprised 10% of the number of *Bythotrephes* in rainbow smelt, 30% in lake herring, and 70% in lake whitefish.

Alpha values calculated for rainbow smelt and lake herring indicated that both species negatively selected or avoided *Bythotrephes* (Table 3). In terms of relative selectivity, lake herring prefered large copepods, while rainbow smelt prefered cladocerans and large copepods equally. Both species avoided small copepods.

According to Spearman rank correlation analyses (Table 4), the number of *Bythotrephes* in the gut significantly increased with length for both rainbow smelt and lake herring. This was also true for lake whitefish when spines and zero values were included in the analysis. Preference for *Bythotrephes* by rainbow smelt and lake herring did not increase with length.

Discussion

We present results from a single sampling date and assume that the abundance of prey in fish stomachs relative to the environment represents typical foraging patterns. Opportunities to sample YOY are relatively rare in Lake Superior. National Biological Survey trawls are conducted every 2 years in Keweenaw Bay and do not always yield a sufficiently large sample size of fish. In addition, although bottom trawls target fish at a specified depth, fish are collected throughout the water column as the trawl descends and ascends, and may not include fish capable of escaping the trawl. Plankton hauls are assumed to represent all available prey species; however, they can also exclude animals able to escape the net. In our case, the plankton net captured few mysids compared with the number that occurred in lake whitefish, but also captured amphipods, an item that did not occur in any of the diets. The net theoretically excluded prey <0.3 mm due to its mesh size. While prey items <0.5 mm were not found in fish stomachs, small prey will be digested faster than large prey. Thus, both plankton sampling and fish digestion may have biased our prey selection estimates in favor of larger items. This, however, should not change our results given that *Bythotrephes* was the larger prey relative to daphnids and copepods. Selection estimates do not change when prey numbers are converted to biomass or volume.

Prey selection indices assume that all prey included in estimates were available or accessible to the predator. To help meet this assumption, we excluded fish smaller than the size of the smallest fish to have ingested a *Bythotrephes*

Fig. 2. Number of *Bythotrephes* including spines found in fish stomachs according to fish length for rainbow smelt (N = 8), lake herring (N = 25), and lake whitefish (N = 21). Only those fish taken from ≥48 m are shown. Zero values are included to illustrate relative consumption by fish.

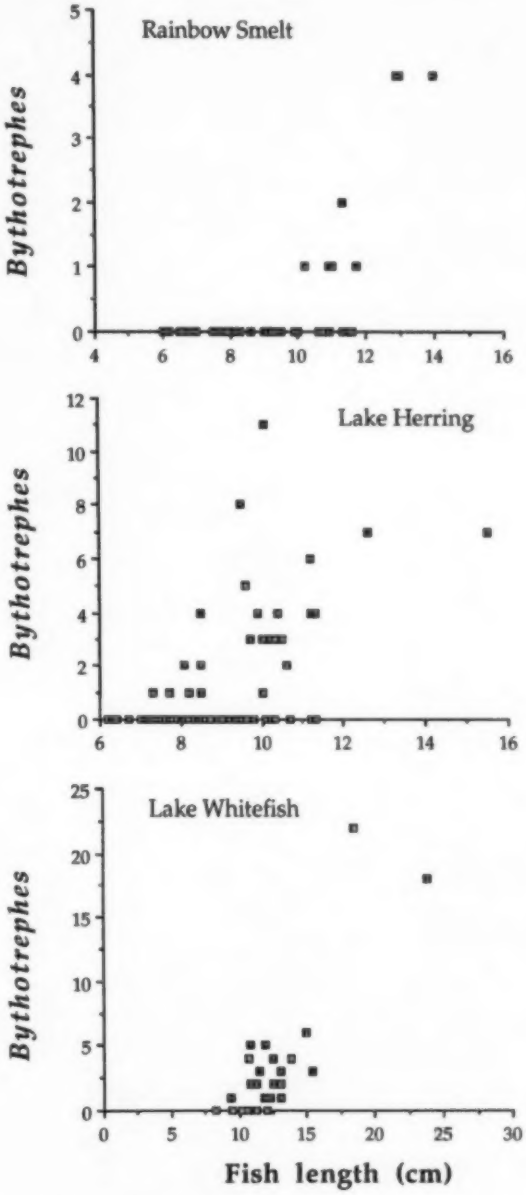


Table 2. Total numbers of zooplankton per tow from the surface to one of three depths estimated by vertical plankton tows.

	Depth (m (SE))		
	0–20	0–30	0–40
Copepoda	1360.0	1431.0	1137.9
Large	56.7 (10.6)	64.3 (15.7)	**37.9** (3.8)
Small	1303.3 (337.6)	1366.7 (213.0)	**1100.0** (282.9)
Cladocera	279.3	365.6	**639.2**
Holopedium	154.0 (38.8)	178.9 (44.5)	194.7 (41.8)
Daphnia	81.0 (16.4)	141.1 (29.5)	372.2 (62.7)
Ceriodaphnia	30.0 (5.1)	40.0 (11.7)	65.6 (8.0)
Bosmina	13.3 (7.0)	0	1.1 (1.1)
Diaphanasoma	1.0 (0.1)	5.6 (2.9)	5.6 (1.1)
Bythotrephes	168.6[a]	167.3[a]	377.6[a]
Three-barb	45.3 (4.9)	70.0 (13.1)	**136.0** (21.4)
	54%,[b] 39%[c]	76%,[b] 19%[c]	61%,[b] 34%[c]
Two-barb	66.0 (22.1)	61.3 (13.8)	**97.3** (35.8)
One-barb	57.3 (25.5)	36.0 (17.2)	144.3 (75.4)
Neonates	1.0 (1.0)	0.3 (0.3)	2.3 (1.3)
Spines	4.3 (1.9)	4.3 (0.9)	12.7 (4.4)
Mysis	0	6.7 (3.3)	0
Diporeia	10.3 (9.0)	0	1.1 (1.1)

Note: The mean of three replicates ± SE is given for each depth. Values in bold were those used to approximate variable n in calculating the Manly–Chesson selectivity index, alpha.

[a]Total excludes neonates and spines.

[b]Percentage of three-barb adults with brood.

[c]Percentage of three-barb adults with resting eggs.

from alpha estimates. In calculating alpha, we used the average number of prey collected by the 0–40 m plankton tows to represent the prey available to predators collected at or below 40 m. However, fish may have restricted their feeding to certain depths. In general, it is difficult to estimate the area in which a fish has foraged in the field. We have assumed that all fish had equal opportunity to encounter *Bythotrephes*. *Bythotrephes* is a conspicuous animal in the plankton due to its large size and dark eye, and it lacks the transparency of other invertebrate predators such as *Leptodora* and *Chaoborus*. Mature females often have large brood chambers filled with dark orange resting eggs, or multiple embryos each with their own dark eye. Although *Bythotrephes* distribution is often patchy, it is not uncommon to find them in a dense layer above the thermocline in stratified waters (D.R. Barnhisel, personal observation). On our sampling date, *Bythotrephes* densities in the 0–20 m plankton tow were similar to those in the 0–30 m tows. Densities increased more than twofold in the 0–40 m tows as we towed over greater depths.

Given that spines without bodies represented a substantial proportion of *Bythotrephes* consumed by lake whitefish, we cannot exclude spines from consumption estimates without the possibility of biasing our results. Evans (1988) first discovered *Bythotrephes* in Lake Michigan in the guts of deepwater sculpin (*Myoxocephalus thompsoni*) and speculated that they were ingested as dead or dying prey at the sediment–water interface. Here, the highest proportion of spines was found in lake whitefish feeding on mysids, a prey associated with sediments, and the highest number of spines was found in the deepest plankton tow, 0–40 m (Table 2). While it is difficult to distinguish whether a fish consumed an intact *Bythotrephes* and digested the body but retained the spine, or consumed the spine as it fell through the water column, there is evidence that alewife (*Alosa pseudoharengus*) retains the spine (Mills et al. 1992; L. Frederick, Wisconsin Sea Grant, Madison, Wis., unpublished data). Excluding spines would have caused *Bythotrephes* alpha estimates to indicate even more negative selectivity.

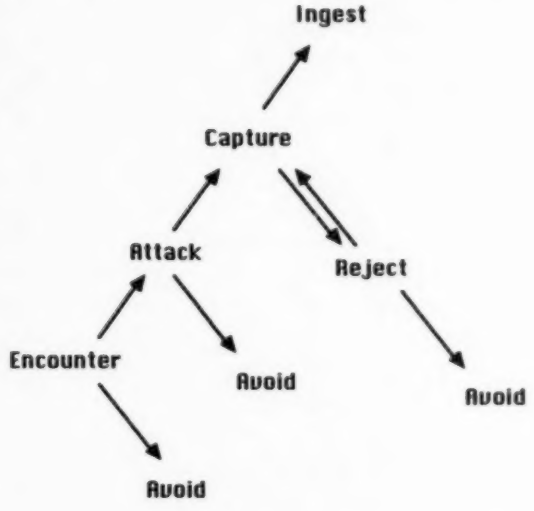
To date, a variety of fish species ≤10 cm have had difficulty ingesting *Bythotrephes* in the laboratory, exhibiting behaviors ranging from rejecting and recapturing the animal many times to whole-body convulsions and learned aversion (summarized in Barnhisel and Kerfoot 1994). Here, we provide evidence that foraging patterns predicted from laboratory experiments may be occurring in the field. For rainbow smelt and lake herring that contained *Bythotrephes* or were theoretically large enough to consume it, we found evidence that they avoided or negatively

Table 3. Alpha values (±SE) indicating positive (+), neutral (n), or negative (−) selectivity for each prey group by fish taken from the depth specified.

Species	Depth (m)	*N*	*Bythotrephes*	Cladocerans	Large copepods	Small copepods
Rainbow smelt	48	15	0.001 (0.000)$^{(-)**}$	0.520 (0.089)$^{(n)}$	0.430 (0.098)$^{(n)}$	0.050 (0.011)$^{(-)**}$
Lake herring	48–53	72	0.002 (0.001)$^{(-)**}$	0.362 (0.037)$^{(-)**}$	0.569 (0.042)$^{(n)}$	0.066 (0.007)$^{(-)**}$

Note: Alpha values were arcsine transformed and compared with the arcsine-transformed value 0.5 using Student's *t*-test (**$p \leq 0.001$). To meet the assumption of prey availability, fish less than the threshold size for *Bythotrephes* consumption were excluded from the analysis. *N* = number of fish.

Fig. 3. Behavioral pathways observed for fish preying on *Bythotrephes* in the laboratory and hypothesized for fish in the field. Depending on fish size and experience, a fish can choose to avoid *Bythotrephes* at a number of junctions.



selected *Bythotrephes* relative to other prey. Although we did not calculate preference for lake whitefish, mysids outnumbered *Bythotrephes* in the gut on average six to one. Many fish larger than the smallest fish to have consumed *Bythotrephes*, and thus potentially capable of ingesting it, did not consume the spined crustacean. For fish greater than or equal to the threshold size for *Bythotrephes* consumption, 21–65% had no *Bythotrephes*. In the laboratory, we have seen up to 50% of YOY yellow perch at one time develop aversion to *Bythotrephes* during feeding trials (Barnhisel 1991*b*).

Consumption increased as fish size increased according to a Spearman rank correlation analysis. One might expect that larger fish with greater gut volumes would contain more *Bythotrephes*. Also, larger fish would encounter and capture *Bythotrephes* more often. Encounter rates increase with swimming speed, and reactive volume (Gerritsen and Strickler 1977) associated with greater visual acuity of larger fish (Hairston et al. 1982). Capture rates may increase with greater buccal volume (O'Brien 1987). Preference, however, did not increase with size in rainbow smelt and lake herring indicating that *Bythotrephes* may continue to be underutilized relative to its abundance even though fish are large enough to ingest it.

Aversion as measured in the laboratory is the decrease in probability over time that a fish will attack a *Bythotrephes* it encounters (Barnhisel 1991*b*). Figure 3 shows the behavioral choices we assume that fish made when they encountered *Bythotrephes*. Fish less than the threshold size for consuming *Bythotrephes* were gape limited and thus not able to ingest the spined crustacean, although they may have attacked and captured it. Those fish greater than or equal to the threshold size for consuming *Bythotrephes* were no longer gape limited, but may have been behaviorally constrained from attacking and ingesting it due to past negative encounters. Thus, although gape limitation may influence the size at which fish can comfortably feed on *Bythotrephes*, a fish's decision to ingest a *Bythotrephes* is most likely related to its size, and previous experience given that it has obtained a sufficient size.

Other field studies provide evidence that fish avoid *Bythotrephes* according to size. Stomach analyses by Guma'a (1978) suggest that fish <1.5 cm in length did not prey on *B. longimanus*, a smaller, more fragile form with an average length of 0.3 cm (Nilsson 1979). According to analyses by Schneeberger (1991) and Baker et al. (1992), *Bythotrephes cederstroemi* was not an important food item for yellow perch <10.0 cm in Lake Michigan. Bur and Klarer (1991) reported the consumption of *Bythotrephes* by yellow perch >12.5 cm and white perch >11.9 cm in Lake Erie. Although Mills et al. (1992) found only *Bythotrephes* tail spines in alewife, the smallest alewife to contain a spine was 7.5 cm. Hartman et al. (1992) found age-0 and age-1 yellow perch and white perch and age-0 gizzard shad (*Dorosoma cepedianum*) having no *Bythotrephes* in the gut, although adult emerald shiner (*Notropis atherinoides*) and spottail shiner (*N. hudsonius*) captured simultaneously in bottom trawls contained *Bythotrephes* (no fish lengths or plankton estimates given.

Sprules et al. (1990) predicted that *Bythotrephes* would not have any major ecological impact in the Great Lakes. However, Lehman (1987) hypothesized that *Bythotrephes* might reduce recruitment of fish that consume the same prey items as *Bythotrephes*, such as *Daphnia*, through resource competition. Barnhisel and Kerfoot (1994) predicted strong indirect exploitative competition interactions between small fish and *Bythotrephes*, with the greatest negative effects on size-classes and species of planktivores too small to utilize the spined zooplankter. However, the effects of *Bythotrephes* on fish populations may be

Table 4. Spearman rank correlation analysis of prey numbers in gut and alpha values for prey (arcsine transformed) according to fish length for all fish greater than or equal to the threshold size at which the species consumes *Bythotrephes*.

Prey numbers/length	Rainbow smelt (N = 15)	Lake herring (N = 72)	Lake whitefish (N = 29)
Bythotrephes (includes spines)	0.488* (N = 17)[a]	0.482**	0.547*
Bythotrephes (excludes spines)	0.617* (N = 17)[a]	0.866**	0.291
Bythotrephes (excludes zero values)	0.849* (N = 8)	0.539* (N = 25)	0.070 (N = 21)
Cladocerans	0.262	0.087	—
Large copepods	0.465	0.409**	—
Small copepods	0.272	0.364**	—
Mysis	—	—	−0.071 (N = 23)

Alphas/length	Rainbow smelt (N = 15)	Lake herring (N = 72)
Bythotrephes (includes spines)	0.065	0.128
Cladocerans	−0.231	−0.169
Large copepods	0.271	0.168
Small copepods	0.206	−0.218

Note: The coefficient r_S was calculated for numbers of *Bythotrephes* including and excluding spines for fish regardless of whether they consumed *Bythotrephes* (includes zero values) and for fish that contained either an intact *Bythotrephes* or spine (excludes zero values). The coefficient r_S was calculated using alpha estimates for *Bythotrephes* that included spines. Significance of r_S: *$p \leq 0.05$; **$p \leq 0.001$. N = number of fish included in each analysis.

[a]Includes two fish in which only the number of *Bythotrephes* in the gut could be accurately determined.

modified if it contributes a substantial proportion to the diets of larger fish. *Bythotrephes* is approximately five times greater in weight than a 2-mm *Daphnia pulicaria* (Barnhisel 1990). It may have contributed up to 3% of the fish diet biomass in Keweenaw Bay assuming that its biomass is 5–10 times greater than a small daphnid or copepod. However, the consequences for Great Lakes fish populations in which YOY life stages are unable to utilize *Bythotrephes* can be severe. Fish in their first year of life are critically dependent on zooplankton and are likely to compete with *Bythotrephes* for that resource. If fish are unable or unwilling to utilize the spined zooplankter, they indirectly affect their own resource availability by allowing *Bythotrephes* populations to go unchecked. Although larger life stages of fish may consume *Bythotrephes*, these fish must first pass through periods in which positive growth is important in decreasing predation risk and and increasing overwintering survival. Thus, while *Bythotrephes* may represent a resource bonus to larger fish, it is likely to cause an overall decrease in small fish survivorship.

Our results relate to the hypotheses regarding whether fish (Sprules et al. 1990) or *Bythotrephes* (Lehman 1988, 1991) may be responsible for the shift in zooplankton composition in Lake Michigan. Sprules et al. (1990) stated that *Bythotrephes* density cannot become high enough to alter zooplankton abundances given its susceptibility to fish predation, and thus, fish are responsible for the zooplankton decline. Lehman and Cáceres (1993) stated that the high numbers of *Bythotrephes* they observed in offshore Lake Michigan are an indication that fish predation is relaxed, and thus, fish cannot be responsible for the observed decline in zooplankton. Our results indicate that researchers should not assume that *Bythotrephes* abundance is tightly regulated by, or an indication of, planktivore abundance, given that some planktivores avoid *Bythotrephes*.

To date, the greatest abundance of *Bythotrephes* (122 individuals·m^{-3}) was reported for eastern Lake Superior in 1988 (Garton and Berg 1990). K. Kirk and C. Kerfoot (Michigan Technological University, Houghton, Mich., unpublished data) have estimated maximum *Bythotrephes* abundances of 18 individuals·m^{-3} in Keweenaw Bay, 15 September 1990. Our results indicate relatively moderate abundances of 48 individuals·m^{-3}. High levels of *Bythotrephes* abundance in Lake Superior relative to other lakes such as Lake Michigan and Lake Erie may be related to the forage fish species composition in Lake Superior, a coregonid-dominated system. Species such as lake herring and lake whitefish have smaller mouths relative to other forage species such as yellow perch and may be more gape limited at similar sizes. The age distribution of *Bythotrephes* was similar to that reported for Scandinavian lakes. All ages are found in upper waters, with greatest numbers above the thermocline (Lindstrom 1955; Lotmarker 1964) and the majority of juveniles in deeper waters (Dzyuban 1979). Physiology, food, and predation each play a role in determining *Bythotrephes* distribution in relation to depth. Juveniles may be found in deeper waters because fish selectively remove them from the lighted upper waters. However, in laboratory studies, yellow perch were no more likely to prey on juvenile *Bythotrephes* given prior negative experiences with adults (Barnhisel 1991*b*).

In summary, we found evidence to support the predictions generated by behavioral experiments (Barnhisel 1991*a*, 1991*b*; Parrish and Margraf 1991) that (1) *Bythotrephes* is not a preferred prey item for young fish, (2) there appears

to be a size threshold at which fish begin to utilize *Bythotrephes* as a food source, and (3) fish are more likely to consume *Bythotrephes* as fish size increases. This study constitutes one of the first diet analyses targeted specifically at juvenile fish and supplemented with plankton samples to estimate selectivity for *Bythotrephes*. We hope to stimulate further diet studies that target the young life stages of fish in determining invasion impacts of *Bythotrephes* in lakes and to illustrate the merits of laboratory experiments to predict what may be occurring in the field.

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Electroporation of sperm to introduce foreign DNA into the genome of loach (*Misgurnus anguillicaudatus*)

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Abstract: An antifreeze protein gene promoter from ocean pout (*Macrozoarces americanus*) linked to a chinook salmon (*Oncorhynchus tshawytscha*) growth hormone cDNA (opAFPGHc) was introduced into loach (*Misgurnus anguillicaudatus*) sperm via electroporation under the following conditions: amplitude, 8 kV; burst time, 1.6 s; number of pulses; 2^8; pulse time, 120 μs; cycle, 6. Compared with the untreated sperm, the fertilization rate driven by the electroporated sperm was decreased slightly, with no significant effect on hatchability. The success rate of gene transfer was 50%. Genomic Southern blotting demonstrated that DNA from transgenic loach contained positive band(s) with higher (37.5%), the same, or lower molecular size as the transferred opAFPGHc fragment. There was a mosaic distribution of exogenous DNA in a wide variety of tissues analyzed. The average weight gain between the 14th and 161th day of the experimental group was 2.5-fold higher than those of the control and mock-treated groups. The body length distribution of the experimental group was rather widely scattered instead of being normally distributed.

Résumé : Un promoteur du gène de la protéine antigel de la loquette d'Amérique (*Macrozoarces americanus*) lié à l'ADNc (opAFPGHc) de l'hormone de croissance du saumon quinnat (*Oncorhynchus tshawytscha*) a été introduit chez des spermatozoïdes d'une loche (*Misgurnus anguillicaudatus*) par technique d'électroporation dont les paramètres étaient : amplitude de 8 kV; salve de 1,6 s; nombre d'impulsions de 2^8; temps d'impulsion de 120 μs et cycle de 6. Comparativement aux témoins, le taux de fécondation des spermatozoïdes traités par électroporation était légèrement plus faible, mais il n'y a pas eu d'incidence sur le taux d'éclosion. Le taux de réussite du transfert génique a été de 50%. Un transfert Southern du génome a montré que l'ADN des individus transgéniques donnait une ou plusieurs bandes positives à taille moléculaire supérieure (37,5%), égale ou inférieure à celle du fragment transféré (opAFPGHc). Il y avait répartition en mosaïque de l'ADN exogène dans les divers tissus examinés. Le gain de poids moyen entre le jour 14 et 161 a été de 2,5 fois supérieur chez le groupe expérimental, comparativement aux groupes témoins ou ayant fait l'objet d'un traitement témoin. La distribution des longueurs des animaux traités n'était pas normale, mais fortement asymétrique.
[Traduit par la Rédaction]

Transgenic animals provide a powerful system for in vivo study of gene regulation, expression, and function. Many approaches have been developed to introduce different types of DNA molecules into zygotes with varying degrees of success. In fish, the most popular methods are (1) microinjection of foreign DNA into oocyte nuclei (Ozato et al. 1986; Tsai et al. 1995), cytoplasm of the developing embryos (Chourrout et al. 1986; Dunham et al. 1987; Zhang et al. 1990), and fertilized eggs (Fletcher et al. 1988; Dunham et al. 1992; Hew et al. 1992; Lu et al. 1992) and (2) electroporation of DNA fragments into eggs (Inoue et al. 1990; Powers et al. 1992; Tsai and Tseng 1994). Electroporation of fertilized eggs could overcome some gene transfer problems in cultured fish, i.e., the invisible pronuclei and tough chorion. However, the foreign DNA might be transferred into the yolk sac of fertilized egg, since the volume of yolk sac is extremely larger than that of germinal vesicle. Furthermore, although the efficiency of gene transfer by electroporation for fertilized eggs is 10–100 times greater than that of microinjection (Powers et al. 1992), transfer efficiency is still not high enough to treat the tremendously large number of eggs spawned within a very short time by cultured fish species.

Use of sperm cells as a vector for introducing foreign DNA in the production of transgenic animals has been attempted recently because it is considered as a "mass" gene transfer. This technique has been successful in mice (Lavitrano et al. 1989), pig (Gandolfi et al. 1989), sea

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urchin (Arezzo 1989), bovine (Gagne et al. 1991; Perez et al. 1991), and fish (Khoo et al. 1992; Muller et al. 1992; Symonds et al. 1994). On the other hand, failure was also reported in mice (Brinster et al. 1989; Gavora et al. 1991), chicken (Gavora et al. 1991), and fish (Chourrout and Perrot 1992). Therefore, reliable techniques for direct sperm-mediated DNA transfer have not been established.

Unlike mammals, fish undergo external fertilization. In general, the number of fish sperm is extremely high, i.e., 3–30 billion/mL (Chao et al. 1986), and they lack an acrosome. Fish sperm is apparently tougher and easier to handle than mammalian sperm. Simply adding water is enough to activate fish sperm. These advantages make fish sperm a suitable material for studying sperm-mediated gene transfer.

The aim of this study was to evaluate the possibility of using fish sperm treated with electroporation as a "mass" gene transfer carrier for loach (*Misgurnus anguillicaudatus*) oocytes. Using a DNA construct containing the ocean pout (*Macrozoarces americanus*) antifreeze protein gene promoter linked to a chinook salmon (*Oncorhynchus tshawytscha*) growth hormone (GH) cDNA (opAFPGHc), the results show that exogenous GH gene can be introduced into loach oocyte by electroporated sperm and generate fast-growing transgenic loach.

Materials and methods

Experimental animals

Gametes for in vitro fertilization were obtained from loach 20–50 g in body weight, purchased locally, and maintained on a natural light and dark cycle.

Egg collection

Mature female fish (over 30 g) were induced to ovulate by intramuscular injection of chorionic gonadotropin (CG): one-third dosage of 15 IU CG/g body weight was injected initially and the remaining two-thirds dosage was injected after 2 h. Eggs were spawned 10–13 h after CG injection. The eggs from a total of 16 female loach were collected, mixed, weighed, and divided into groups for one trial. In total, we used 22.5, 8.4, and 23 g of eggs (approximately 3000 eggs/g) in the experimental, mock-treated, and untreated control groups, respectively.

Sperm preparations

Testes were obtained from mature male loach (over 25 g) or loach injected with 10 IU CG/g body weight for 10–13 h. Testes were mixed with 1 mL of PBS solution (0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, and 0.024% KH_2PO_4 adjusted to pH 7.4 with HCl), and homogenized in a glass homogenizer for 1 min at room temperature. The homogenates without tissue debris were pooled together. They were equally divided into several Eppendorf tubes and then centrifuged at 16 000 × *g* for 30 s at room temperature. The sperm suspensions were prepared by resuspending pellets in a final concentration of 1–2 × 10^8 cells/mL electroporation solution (PBS saturated with $CaCl_2$).

DNA preparation

The opAFPGHc fragment, which consists of the antifreeze protein gene promoter of ocean pout fused with the 5′-untranslated region, coding region, and 3′-untranslated region of chinook salmon GH cDNA (Du et al. 1992), was recovered by *Eco*RI digestion of the plasmid opAFP-GHc. This DNA fragment was resuspended in electroporation solution and added to the sperm suspension to a final concentration of 1 μg/mL.

Electroporation (Baekonization)

A noncontact mode instrument (Baekon 2000, Calif.) was used to carry out electroporation. The conditions for electric field strength were varied from 1 to 12 kV, burst time from 0.6 to 2.2 s, number of pulses per cycle from 2^4 to 2^{13}, and number of cycles from 2 to 14. The other three variables were kept constant: pulse time, 120 μs; electrode distance, 1 mm; solution volume, 100 μL in a 1-mL cuvette. In total, 960 different combinations of conditions of amplitude, burst time, number of pulses, and cycles were set for electroporation. Their resultant influences on sperm mobility were examined microscopically and categorized by following the description of Guest et al. (1976).

One hundred microlitres of diluted sperm suspension (10^7 cells) with 0.1 μg of opAFPGHc in electroporation solution (experimental group) or with electroporation solution alone (mock-treated group) were used for electroporation. The control group consisted of sperm without treatment.

DNA analysis of sperm treated with DNase

After 100 μL of diluted sperm suspension was mixed with 0.1 μg of opAFPGHc in electroporation solution, four different concentrations (0, 0.1, 1, and 10 μg) of DNase (Promega) were added, respectively, and then incubated at 37°C for 1 h. Each treatment was duplicated. The genomic DNA of sperm was extracted and analyzed by dot blot using a digoxigenin-labelled (Boehringer Mannheim Biochemica, Germany) opAFPGHc probe to optimize the concentration of DNase capable of completely digesting opAFPGHc persisting outside the sperm.

After complete digestion by DNase, the electroporated sperm were washed several times in PBS solution and their genomic DNA analyzed by Southern blot using a digoxigenin-labelled opAFPGHc probe. Direct incubation of loach sperm in electroporation solution containing opAFPGHc for 30 min at room temperature was also studied. The genomic DNA of the incubated sperm was assayed after DNase digestion and PBS washing.

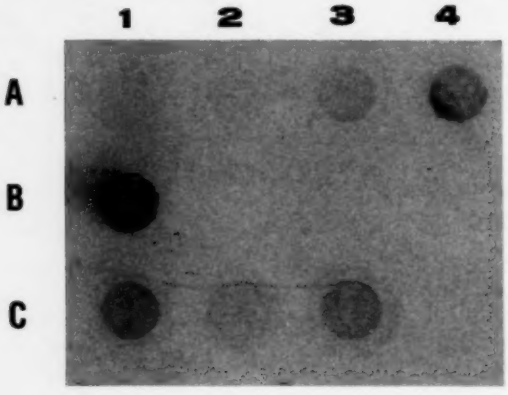
In vitro fertilization

Sperm electroporated in the presence of opAFPGHc (experimental group), sperm electroporated in the absence of opAFPGHc (mock-treated group), and sperm with no treatment (control group) were used to fertilize eggs for 1 min in amounts of around 39 000, 24 000, and 45 000 eggs, respectively, for one trial; another trial had 30 000 and 24 000 eggs in the experimental and control groups, respectively. After fertilization, the eggs were washed twice with water and then dispersed on a net at 28°C for hatching.

Genomic DNA extraction

The genomic DNA was extracted from sperm, whole bodies of 2-week-old fry, or various tissues (red blood cells, brain,

Fig. 1. Dot blot analysis of genomic DNA isolated from electroporated and DNase-treated sperm. Wells of A1, A2, A3, A4, and B1 were standards containing 50, 100, 300, and 500 pg and 5 μg opAFPGHc, respectively, not derived from electroporated sperm; B2, B3, and B4 were 100, 300 and 500 pg of negative control plasmid (pmiwZ); C1, C2, and C3 were genomic DNA isolated from electroporated sperm in the presence of opAFPGHc; C4 was genomic DNA from electroporated sperm in the absence of opAFPGHc.



eye, fin, gill, intestine, muscle, ovary, or testis) of 6-month-old juveniles by following the SDS–phenol method (Inoue et al. 1990).

Dot and Southern blot analyses

Genomic DNA of sperm or 2-week fry was extracted and 1 μg was spotted on a nylon membrane (Amersham). After air-drying, the DNA was cross-linked to the membrane by UV irradiation and then hybridized to a ^{32}P-labelled opAFPGHc. For Southern blot analysis, genomic DNA was digested with *Eco*RI, electrophoresed on a 0.8% agarose gel, transferred to a nylon membrane, and then hybridized to a ^{32}P-labelled opAFP fragment (obtained by digesting a opAFPGHc fragment with *Eco*RI and *Pst*I), 2.2 kb, not containing GH cDNA. Prehybridization was carried out in a solution of 50% formamide, 5× Denhart's solution, 0.1% SDS, and 6× SSC (1× SSC: 0.15 M NaCl and 15 mM sodium citrate) at 42°C overnight. Hybridization was carried out in the same solution but containing a denatured probe at 42°C overnight. Membranes were washed twice in a solution containing 1× SSC and 0.1% SDS for 15 min at 42°C and twice in a solution of 0.1× SSC and 0.1% SDS for 30 min at 68°C before autoradiography.

PCR analyses

Four oligonucleotide primers (Du et al. 1992) were synthesized by Promega for PCR analysis: primer A, 5′-GTCAGAAGTCTCAGCTACAGC-3′ (nucleotides 27–47); primer B, 5′-ATCTCAACAGTCTCCACAGGT-3′ (nucleotides 861–881 of antisense strand); primer C, 5′-TCTGCTGATGCCAGTCTTACT-3′ (nucleotides 161–181); and primer D, 5′-ACAGAAGTCCAGCAGGAATAT-3′ (nucleotides 339–359 of antisense strand). The genomic DNA samples were digested with *Eco*RI and denatured by boiling for 10 min. PCR was carried out in 100 μL of a solution consisting of 20–30 ng templates, 10 pmol of each primer, 200 μM of each dNTP, 10 μg of bovine serum albumin, 1.75 mM $MgCl_2$, and 1 U of Taq DNA polymerase in a 1× PCR buffer (Promega). Amplification was performed using a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). PCR consisted of 25 denaturing cycles at 92°C for 1 min, annealing at 50°C for 2 min (the first five cycles at 60°C), and extension at 72°C for 3 min with a 10-min extension at 72°C. Each PCR sample was subjected to electrophoresis on a 3% NuSieve GTG agarose gel (FMC BioProducts, U.S.A.).

Growth observation

Fifty loach fry from each group were kept separately in an aquarium (70 × 45 × 15 cm^3) at 28°C. Rotifer (*Brachionus* spp.) was used to feed fry aged 1–5 days after hatching, R-1 microfeed (Japan) for fry aged 5–12 days, and Tetra (crude protein 46%, crude fat 7%, crude fiber 5%, and moisture 8%, Werke) after the 12th day. Fish were fed twice a day at 5% of total weight. Body weight and length were measured individually from 35 randomly selected loach from each group. Growth rate (percentage) was calculated by $(WT - wt)\cdot wt^{-1}\cdot 100$ where WT and wt are the final and initial weight, respectively (Takeuchi 1988).

Results

Electroporation conditions and sperm activity

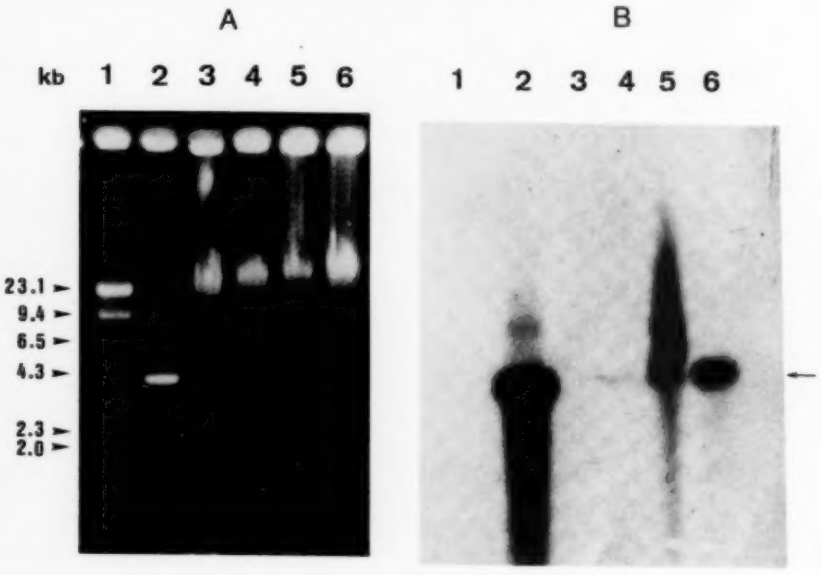
Amplitude, burst time, number of pulses, and cycles were the four variables examined for their effect on sperm mobility with a pulse time kept constant at 120 μs. The most influential variable for sperm mobility was amplitude. Sperm mobility was gradually lost when the amplitude was increased over 8 kV and the other variables were kept at a minimum (burst time 0.6 s, number of pulses 2^4, and cycles 2). If 10 kV was used to treat sperm, their mobility was totally lost or only Brownian movement was detectable.

After examining 960 combinations of amplitude, burst time, number of pulses, and cycles, the following conditions are useful for loach sperm electroporation: amplitude from 5 to 8 kV, burst time from 0.6 to 1.6 s, number of pulses per cycle from 2^5 to 2^8, and number of cycles from 4 to 6 under 120 μs pulse time in a solution volume of 100 μL contained in an 1-mL cuvette with 1-mm electrode distance.

Based on the excellent uptake of foreign DNA and normal activity of sperm, conditions used for sperm electroporation in all subsequent experiments were the following: amplitude 8 kV, burst time 1.6 s, number of pulses 2^8, and cycles 6. The activity of the electroporated sperm remained the same as that of the nonelectroporated sperm.

The mobility of sperm was examined from 20 males, whose gonadosomatic index (GSI, the percentage of gonadal weight to total weight) ranged from 0.25 to 1.28%. We found that sperm motility was extremely poor under the electroporation conditions set as above if GSI was less than 0.75%. Thus, sperm used for this study was obtained from males whose GSI was between 0.75 and 1.28%.

Fig. 2. Southern blot analysis of genomic DNA isolated from sperm. (A) Genomic DNA run on the gel; (B) transferred to a membrane and hybridized to opAFPGHc probe. Lane 1, molecular marker, *Hin*dIII-digested lambda; lane 2, 500 ng of opAFPGHc (positive control); lane 3, sperm incubated with water; lane 4, sperm incubated with opAFPGHc solution for 30 min; lanes 5 and 6, sperm electroporated with opAFPGHc. Arrow in Fig. 2B indicates the molecular size of electroporated DNA (4 kb).



DNA uptaken by sperm after electroporation

After digestion with 10 μg of DNase at 37°C for 1 h, no positive reaction was found on dot or Southern blot analysis of sperm suspension (100 μL) mixed with opAFPGHc (0.1 μg). However, positive reactions were found for groups treated with 0 (control), 0.1, and 1 μg of DNase.

After the diluted sperm suspensions were electroporated in the presence of opAFPGHc, the suspension was treated with 10 μg of DNase at 37°C for 1 h. The genomic DNA of pooled sperm (10^7 cells) was extracted and analyzed by dot blot hybridization. Results showed that DNA isolated from the electroporated sperm with opAFPGHc was positive (C1, C2, and C3 of Fig. 1) whereas DNA isolated from the electroporated sperm in the absence of opAFPGHc was negative (C4 of Fig. 1).

Southern blot analysis of genomic DNA isolated from the electroporated sperm in the presence of opAFPGHc showed a positive band with a molecular weight (MW) of 4 kb, which was the same size as that of opAFPGHc (lanes 5 and 6 of Fig. 2). Compared with the band intensity of 500 ng of opAFPGHc, 50 ng of transgene was electroporated into 10^7 sperm (lanes 2 vs. 6 of Fig. 2). Thus, on average, approximately 1000 molecules of opAFPGHc were carried by each transferred sperm. However, the genomic DNA obtained from the electroporated sperm in the absence of opAFPGHc (mock-treated group) was negative (lane 3 of Fig. 2). Moreover, sperm incubated with opAFPGHc without electroporation treatment were also negative (lane 4 of Fig. 2).

Table 1. Fertilization and hatching rate of loach.

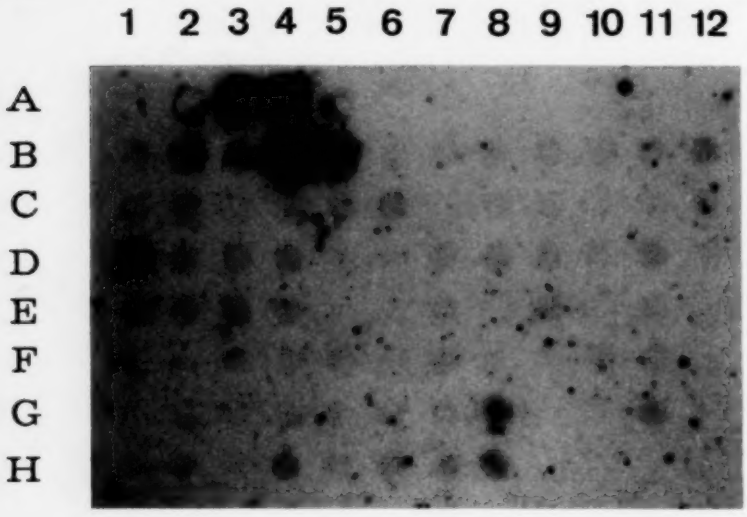
	I		II		
Trial	C	E	C	C1	E
Egg weight (g)	8.2	9.6	14.8	8.4	12.9
Fertilized rate (%)	90	62	88	76	72
Hatching rate (%)	71	67	72	68	61

Note: C, control group (sperm without treatment); C1, mock-treated group (sperm electroporated without foreign DNA); E, experimental group (sperm electroporated with opAFPGHc). One gram of eggs contains approximately 3000 oocytes.

Effect on fertility and hatchability of electroporated sperm

Compared with the untreated sperm (control group), the fertility of electroporated sperm in the absence of DNA fragments (mock-treated group) showed a slight decrease, i.e., 88 vs. 76%, but not much difference in hatchability, i.e., 72 vs. 68% (column C vs. C1 under trial II in Table 1). Compared with the control group, the fertility of electroporated sperm in the presence of opAFPGHc (experimental group) was also lower, i.e., 90 vs. 62% (trial I) and 88 vs. 72% (trial II). Hatchability, on the other hand, did not appear to be significantly different between the control and experimental groups, i.e., 71 vs. 67% (trial I) and 72 vs. 61% (trial II).

Fig. 3. Dot blot hybridization of genomic DNA isolated from 2-week-old loach fry. A1 to A4 were 50, 200, 500, and 1000 pg of opAFPGHc, respectively; A5 to A7 were 200, 500, and 1000 pg of negative control plasmid (pmiwZ); A8 to H8 were 85 loach randomly chosen from the experimental group; H9 to H12 were loach chosen from the untreated control group.



Analysis of transgenic loach by dot, Southern blotting, and PCR

Genomic DNA was extracted from 2-week-old loach fry and analyzed by dot blot hybridization. The success rate for opAFPGHc gene transfer was 52.2% (94 positive out of 180 fry examined) (Fig. 3). When 26 genomic DNA randomly chosen from 94 dot-positive ones were digested with *Eco*RI, run on the agarose gel (Fig. 4A), and analyzed by Southern blotting (Fig. 4B) using the promoter region of antifreeze protein gene as a probe, 92.3% (24 out of 26) of samples were identified as positive reaction. A 4-kb positive band (same size as the transgene) was shown on 58% (14 out of 24) of the transgenic fry loach (as lane 7 of Fig. 4B). Nevertheless, different-size band(s) combined with 4 kb were observed: >23, 8, and 4 kb (lanes 11 and 17 of Fig. 4B); >23 and 4 kb (lanes 10 and 16 of Fig. 4B); 8, 4, and 2.4 kb (lanes 12 and 15 of Fig. 4B); and 4, 2.4, 0.7, and 0.4 kb (lane 9 of Fig. 4B). Furthermore, a single positive band of 8 kb was also found in a transgenic fry (lane 13 of Fig. 4B). In all, the transgene was integrated into the genome of 37.5% (9 out of 24) of the transgenic loach as indicated by the positive band(s) of MW higher than the transgene, 4 kb (Fig. 4B).

The oligonucleotide pair sets, primers A/B, C/D, and A/D, chosen for PCR amplification generated 855, 199, and 333 bp, respectively (Du et al. 1992). As expected, all three PCR products were observed (lanes 4 and 5 of Figs. 5A and 5C). On the other hand, when primer sets used for DNA either from the nontransgenic loach in the experimental group (lane 3 of Fig. 5A and 5C) or from the control loach (lanes 1 and 2 of Fig. 5A and 5C), no PCR product was produced. The PCR products were also positive to Southern blot hybridization when opAFPGHc was used as a probe (Fig. 5B).

Genomic DNA was isolated from 6-month-old loach randomly chosen from the experimental and control groups, digested with *Eco*RI, and analyzed by Southern blot hybridization: 5 out of 11 blood samples obtained from the experimental group showed a positive band, with a MW of 4 kb, the same as that for transferred DNA (lanes 1, 2, 3, and 4 of Fig. 6), and with MW higher than 4 kb, i.e., 8 and >23 kb (lane 29 of Fig. 6). However, this positive pattern did not occur in blood samples from nontransgenic loach in the experimental group (lanes 5, 6, and 30–33 of Fig. 6) or from the control group (lanes 10–27 of Fig. 6).

Mosaicism

Genomic DNA from various tissues of four males and four females obtained from the experimental group was analyzed by using PCR techniques (Table 2). Except for one loach (M3) which was negative, a 199-bp positive band was found on the gel from at least one of the tissues, but not all tissues. A fin- or blood-positive individual did not necessarily mean that other tissues would be positive. This indicated that the transgenic loach had a mosaic pattern.

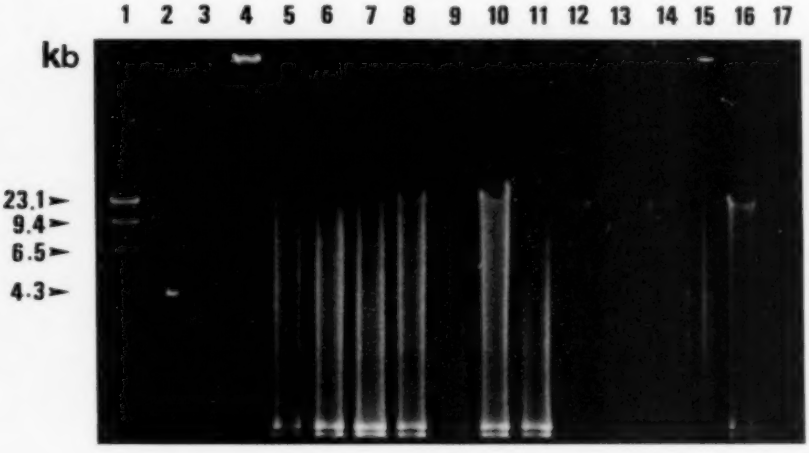
Genomic DNA isolated from various tissues of F2 was further analyzed by Southern blotting. Results showed that (1) a positive band of 4 kb was found in eyes (lane 5 of Fig. 7), (2) positive bands of 4 and 8 kb were found in ovary (lane 8 of Fig. 7), (3) a positive band of higher than 4 kb (>23 kb) was found in brain (lane 9 of Fig. 7), and (4) no positive band was found among muscle, kidney, liver, fin, gill, intestine, and blood (lanes 1–4, 6, 7, and 10 of Fig. 7, respectively).

Growth rate and weight gain

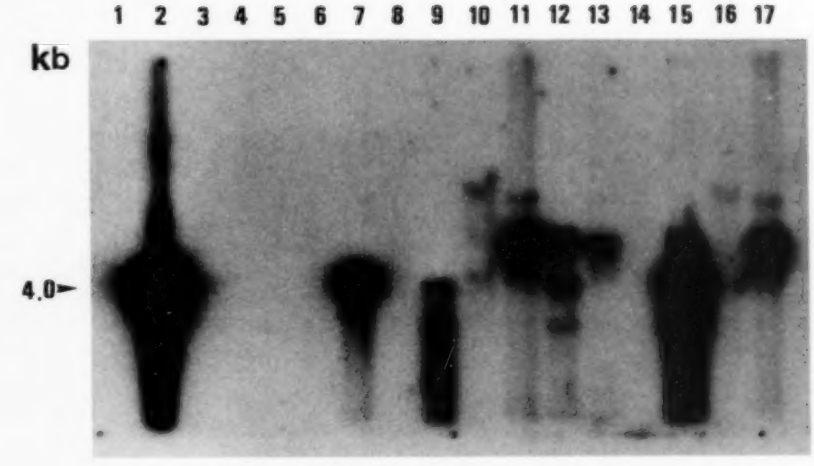
There appeared to be no difference in growth rate between loach treated with the electroporated sperm not containing

Fig. 4. Southern blot analysis of presumptive transgenic 2-week-old loach developed from electroporated sperm carrying opAFPGHc. (A) *Eco*RI-digested genomic DNA was electrophoresed on a gel; (B) Southern blot hybridization. Lane 1, molecular marker in kilobase pairs (kb); lane 2, opAFPGHc (positive control) used for transferring; lane 3, blank; lanes 4 to 17, different presumptive transgenic individuals. Arrowhead in Fig. 4B indicates the molecular size of the transgene (4 kb).

A



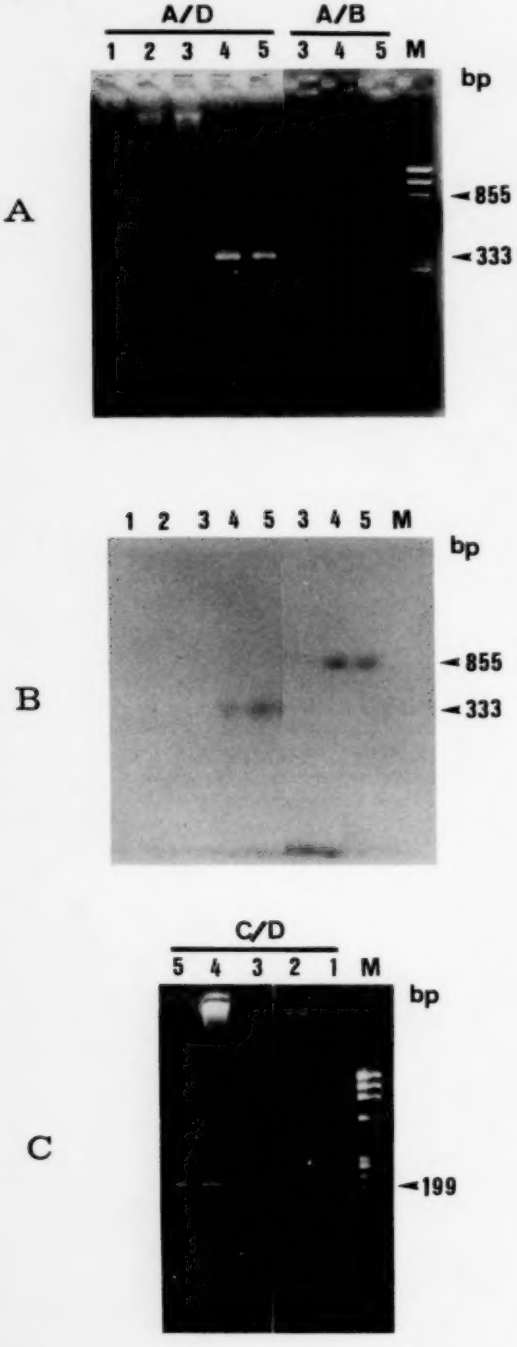
B



opAFPGHc (mock-treated group) and those treated with control sperm (control group) (Fig. 8). However, the growth rate of loach treated with electroporated sperm carrying opAFPGHc (experimental group) showed significant enhancement starting by the 100th day, compared with that of the above control groups.

The mean body weight of 2-week-old loach from the control and experimental groups was 0.01 ± 0.002 g. By the

Fig. 5. PCR analysis of genomic DNA isolated from 2-week-old loach. Lanes 1 and 2, from untreated control; lanes 3, 4, and 5, from the experimental group; lane M, molecular markers, *Hae*III-digested ϕX-174-RF DNA. (A) PCR analysis using primers A/D and A/B; (B) Southern blot analysis of the PCR product from Fig. 5A using opAFP GH cDNA as a probe; (C) PCR using primers C/D.



70th day, the mean body weight had increased to 0.25 ± 0.13 and 0.45 ± 0.33 g for the control and experimental groups, respectively. By the 161st day, it had increased to 1.95 ± 0.99 and 4.48 ± 2.88 g for the control and experiment groups, respectively. The weight gain was 194 and 447% for the control and experimental groups, respectively, during the period from the 14th to the 161st day after hatching.

Body length – frequency distribution

The initial body length of the loach after hatching was around 2.99 ± 0.02 mm. At the 70th day, it averaged 30.62 ± 4.58 and 35.97 ± 9.42 mm for the control and experimental groups, respectively (Fig. 9). At the 161st day, they had grown to an average of 66.56 ± 10.06 and 81.57 ± 21.44 mm for the control and experimental groups, respectively. Compared with the control group, the size distribution for the transgenic group was rather widely scattered.

Morphological abnormalities

Different types of morphological abnormalities were found in 30% of loach in the experimental group, i.e., abnormal body shape (curl or V-shape), shortened operculum, absence of body segment between anal and tail fins, degenerated fin (pectoral, dorsal, and (or) pelvic fins), growth retardation (i.e., the smallest size indicated on Fig. 9), early maturation, and improper location of organs. However, less than 5% of loach in the mock group and less than 0.05% of loach in the untreated group were abnormal.

Discussion

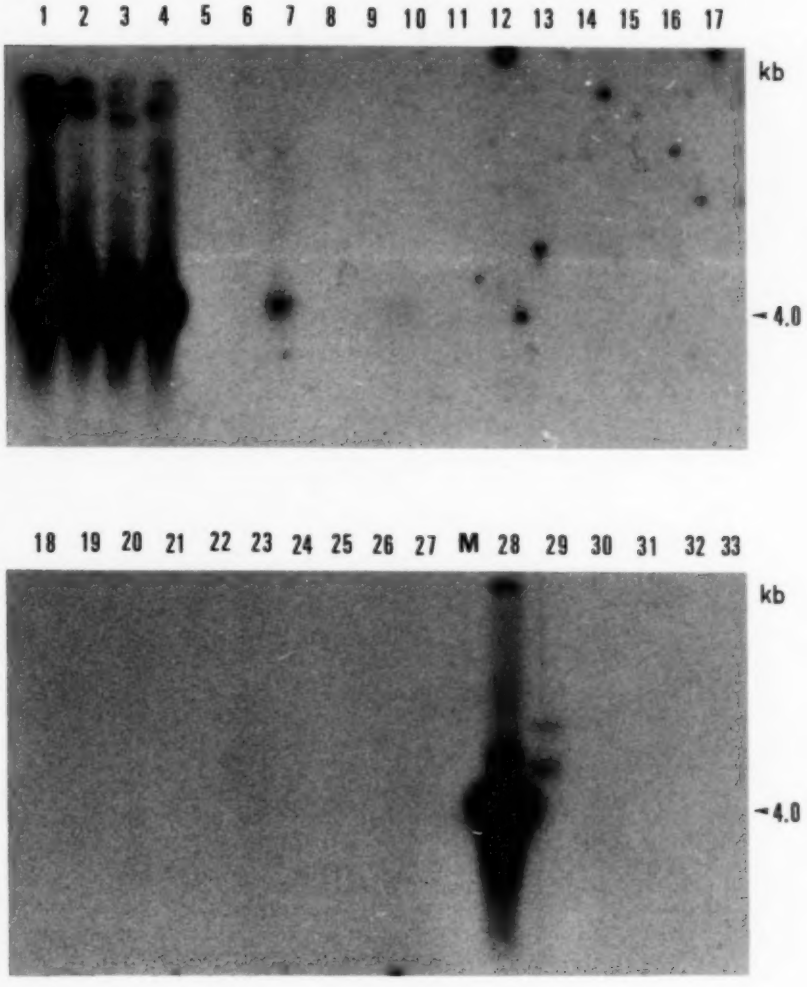
Keeping normal sperm mobility of loach after electroporation is dependent on the maturity of the male and the conditions of electroporation. We suggest that sperm be obtained from males whose GSI is at least higher than 0.75%. Conditions of electroporation should not be too severe, no higher than 8 kV for amplitude, 1.6 s for burst time, 2^8 for number of pulses, and 6 for cycles.

The fertility of the electroporated sperm (mock-treated group and experimental groups) was slightly less than that of untreated sperm. We observed that some sperm fused together occasionally after electroporation, although the motility of sperm did not show any difference under microscopic observation after electroporation. We propose that those fused sperm might have caused lower fertility.

Similar to the control group, cumulative mortality of the mock-treated group was around 20–30%. However, the mortality of the group hatched using electroporated sperm treated with opAFPGHc was comparatively high, i.e., 40–50%. Because the mortality of the mock-treated group was close to that of the control group, the higher mortality that occurred in the experimental group may have been due to the random integration of transferred gene and (or) the expression of the exogenous GH gene.

Since 0.1 μg of opAFPGHc in sperm suspension prior to electroporation could be completely digested by 100 μg DNase/mL, the positive hybridization found in the dot and Southern analyses (Figs. 1 and 2) suggests that foreign DNA was introduced into sperm using the electroporation

Fig. 6. Southern blot analyses of *Eco*RI-digested genomic DNA isolated from red blood cells of 6-month-old loach. Lanes 1 to 6, and 29 to 33, from the experimental group; lane 7, the transgene opAFPGHc DNA fragment; lanes 8 to 27, from the control group; lane 28, plasmid containing opAFPGHc; lane M, molecular markers (*Hin*dIII-digested lambda). Arrowheads indicate the molecular size of the transgene (4 kb).



conditions outlined in this study. Our findings are in agreement with the results of Gagne et al. (1991), who demonstrated that foreign plasmid could be introduced into bovine oocytes by electroporated spermatozoa. However, if sperm was incubated with opAFPGHc, the exogenous DNA was not transferred into sperm (lane 4 of Fig. 2). This result was consistent with that of Gavora et al. (1991), who failed to transfer DNA into eggs of mice and chicken by using spermatozoa incubated with bacterial plasmid DNA. The result was also in agreement with Chourrout and Perrot (1992), who failed to detect transgenesis in rainbow trout by incubated sperm. These observations strongly support Muller et al. (1992), who demonstrated that a foreign gene was introduced into fish with electroporated sperm, but not with incubated sperm. However, it was contrary to the report of Khoo et al. (1992), who suggested that foreign plasmid could be introduced into zebrafish by simply using sperm incubated with DNA. Therefore, the exact mechanism for sperm-mediated DNA transfer needs further study.

Three oligonucleotide pairs were chosen for PCR amplification to identify transgenesis of the transferred chinook salmon GH cDNA into loach. The results showed that primers and PCR conditions were used to amplify the exogenous GH cDNA, but not for the endogenous GH gene. However, Du et al. (1992) reported that using C/D primers showed the presence of two bands. One band (199 bp), derived from the cDNA of the injected plasmid, and another band (344 bp) were amplified from the genomic DNA of the host fish. The latter band was used as an internal positive control of Atlantic salmon. Unlike Du et al.'s (1992) result,

Fig. 7. Southern blot analysis of *Eco*RI-digested genomic DNA isolated from the various tissues of a 6-month-old transgenic loach, F2 (lanes 1–10), and a nontransgenic loach (lanes 12–15). Lanes 1 and 12, muscle; lane 2, kidney; lane 3, liver; lane 4, fin; lanes 5 and 13, eye; lane 6, gill; lane 7, intestine; lanes 8 and 14, ovary; lanes 9 and 15, brain; lane 10, blood; lane 11, the transgene opAFPGHc fragment; lane M, molecular marker. The arrowhead indicates the molecular size of the transgene (4 kb).

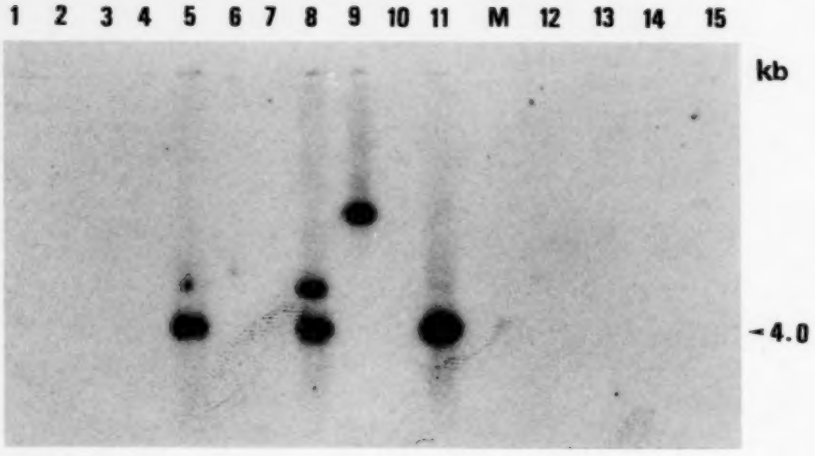
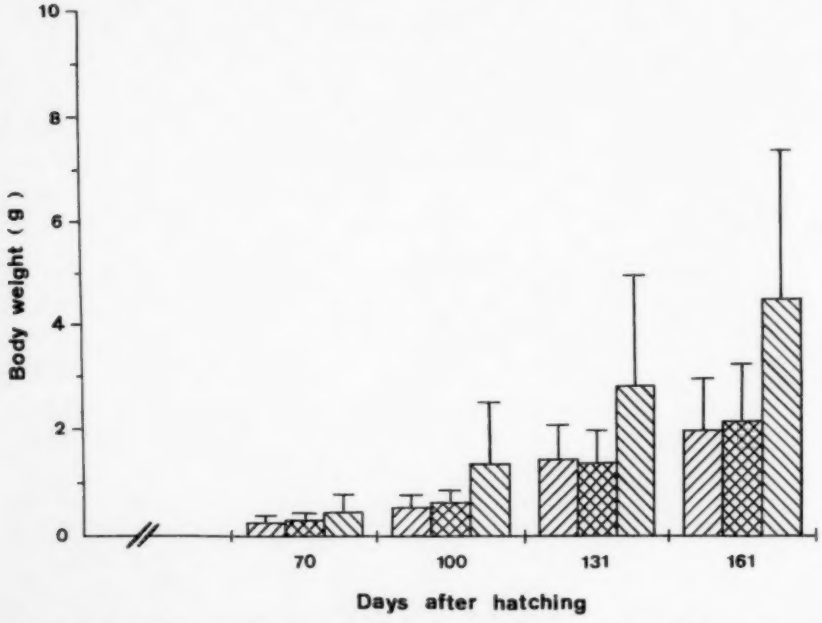


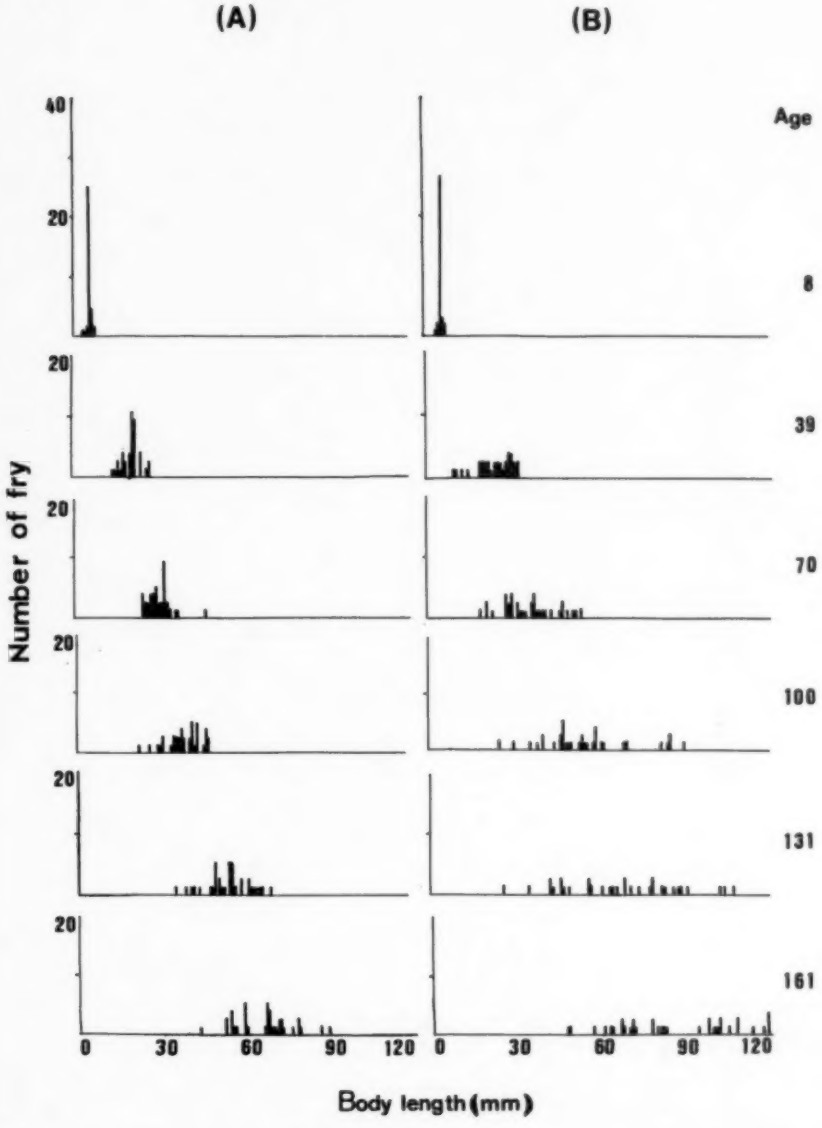
Fig. 8. Body weight (mean ± SE in grams) of 35 loach randomly chosen from the control (left-hatched bars), mock-treated (cross-hatched bars), and experimental groups (right-hatched bars) beginning at the 70th day after hatching.



there was only one expected band, with a size of 199 bp, shown on the gel using C/D primers to detect transgenic loach. This indicated that these primers did not have sufficient sequence identity to anneal to the loach GH gene. Primers were designed for detection of these gene constructs in salmonids. Thus, these primers were useful in detecting exogenous chinook salmon GH cDNA transferred into the loach genome.

Southern blot analysis showed that a positive band with the same MW length as the transferred opAFPGHc fragment (4 kb) occurred in 58% of the transgenic fish examined. Moreover, the band(s) with a MW higher than 4 kb, such

Fig. 9. Monthly body length distribution of 35 loach randomly chosen from the (A) control and (B) experimental groups. Numbers on the right indicate the age of fry in days.



as >23 and 8 kb, were also observed in 37.5% of transgenic loach (Figs. 4, 6, and 7). This evidence strongly suggests that the transferred gene may be integrated into the chromosome(s) of loach after gross rearrangement of DNA molecules. These results agree with those of Inoue et al. (1990), who reported that positive bands with the same size and larger than that of the transferred DNA occurred in transgenic medaka.

Similar to the results from the parental transgenic fish produced by microinjection (Zhu et al. 1989; Du et al. 1992; Dunham et al. 1992), transgenic loach examined in this experiment showed a mosaic pattern (Table 2), although the gene transfer was mediated by the electroporated sperm. Similarly, mosaic distribution of the exogenous δ-crystalline gene in transgenic medaka was also reported, although medaka transgenesis was carried out in oocyte nuclei 8–10 h prior to fertilization (Ozato et al. 1986). However, we found that the transgene was present in gonads in 25% (one out of four males and one out of four females) of the experimental loach (Table 2) and that it existed both in 4- and 8-kb lengths (Fig. 7). Moreover, the form(s) of the transgene in an individual transgenic loach might be mosaic or tissue dependent: a positive band of >23 kb was found in brain, positive bands of 8 and 4 kb

Table 2. PCR analysis of genomic DNA isolated from the various tissues of 6-month-old loach.

	Individual							
Tissue	M1	M2	M3	M4	F1	F2	F3	F4
RBC	−	+	−	−	−	−	−	+
Brain	+	+	−	−	+	+	+	+
Eye	+	−	−	+	+	+	−	−
Fin	−	−	−	+	−	−	+	−
Gill	+	−	−	+	−	−	−	−
Gonad	+	−	−	−	−	+	−	−
Intestine	+	−	−	−	+	−	−	−
Muscle	−	−	−	+	−	−	−	+

Note: Genomic DNA was prepared from loach treated with electroporated sperm carrying opAFPGHc. +, using primers C/D, a DNA fragment with size of 199 bp shown; −, using primers C/D, no 199 bp DNA fragment shown. M, male; F, female; RBC, red blood cells.

were found in ovary, and a band of 4 kb was found in eyes (Fig. 7).

Genomic DNA extracted from fin or red blood cell is routinely used to detect the presence of a transgene sequence in presumptive transgenic fish. However, when genomes were extracted from various tissues, we found that tissue(s) other than fin or red blood cell of some fin- or blood-negative transgenic loaches showed the presence of a transgene (Table 2). This implies that one may underestimate the efficiency of transgenesis by simply examining one tissue, i.e., fin or red blood cell samples, to identify transgenic fish. In addition, results based on genomic DNA examination from fin or red blood cells alone do not imply that the individual must be germ line positive and transmit its transgene to progeny.

Although we have not yet analyzed all the experimental loach, the rate of successful gene transfer that we found in 2-week-old fry was approximately 50%. It was close to that reported for electroporation in zebrafish embryos, i.e., 35–75% (Powers et al. 1992). Nevertheless, the success rate of this study was not only higher than that reported for microinjection in medaka, i.e., 16–27% (Ozato et al. 1986; Inoue et al. 1990; Lu et al. 1992), zebrafish, common carp, and channel catfish, i.e., 0–35% (Powers et al. 1992), but also higher than that reported for electroporation in medaka eggs, i.e., 4–20% (Inoue et al. 1990; Lu et al. 1992) and for electroporation in sperm of carp, tilapia, and catfish, i.e., 2.6–4.2% (Muller et al. 1992). The reasons why our success rate was superior to that of Muller's (1992) report might be due to the differences of electroporation condition, equipment, and fish used.

The transfer efficiency of our approach was much greater than that reported not only for microinjection in oocyte or fertilized egg of medaka, i.e., 1–4 eggs/min (Ozato et al. 1986; Inoue et al. 1990), but also for electroporation in fertilized eggs of zebrafish, i.e., 35–200 eggs/min (Powers et al. 1992). Although survival was comparatively low in this experiment, the sperm-mediated gene transfer was efficient enough to enable processing of several hundred thousand eggs at one time.

Abnormalities of transgenic animals have been reported in mice, such as limb deformity (Woychik et al. 1985) and leglessness (McNeish et al. 1988). Surprisingly, many different types of morphological abnormalities were found in this study. Compared with the untreated control group, the percentage of abnormalities derived from the electroporated sperm was high, especially for the experimental group that carried the transgene opAFPGHc. We suggest that these abnormalities might have been caused by (1) too severe electroporation conditions, (2) too high level of GH resulting from too many copies of foreign GH cDNA and (or) from too strong expression of exogenous GH cDNA, and (3) gene disruption. Therefore, further studies on which factor is involved in causing abnormal transgenic loach would be interesting.

Electroporation has often been used for gene transfer into culture cells and embryos. In this paper, we have clearly demonstrated that foreign genes can be transferred into fish embryos by using electroporated sperm as a vector. Compared with conventional microinjection and electroporation for fish eggs, electroporation of fish sperm is a relatively simple mass gene transfer procedure. The gene construct opAFPGHc does promote growth in transgenic loach, which agrees with the earlier observation by Du et al. (1992), who reported a four- to sixfold growth enhancement in transgenic Atlantic salmon generated using the same gene construct. However, considering the 30% of deformity in this study, it might be advisable to lower the GH level by fine tuning the gene construct to suit the purpose of producing transgenic loach.

Acknowledgements

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Natural variability and the estimation of empirical relationships: a reassessment of regression methods[1]

Yves T. Prairie, Robert H. Peters, and David F. Bird

Abstract: Ecologists often rely on empirically defined statistical relationships to infer how variables might be related. However, the usual method of estimating such relationships (ordinary least-squares (OLS)) is generally inappropriate because of the substantial natural variability of most ecological variables. Natural error variability in the regressor variable can artificially create a significant empirical trend where no underlying or structural relationship exists, or fail to reveal a true structural relationship. In multivariate relationships, natural variability in one variable can induce statistical significance in collinear variables even if they bear no structural relationship. We propose a simple new method, based on instrumental variables, to detect and quantify natural error variability in the regressor variables and to estimate the parameters of the structural relationship. We apply this method to two examples: (1) we show that the structural relationship between adenosine triphosphate concentration (total planktonic biomass) and chlorophyll concentration (autotrophic biomass) does not vary latitudinally in the Southern Ocean despite a significant increase in the OLS slope relating the two at more southerly stations and (2) we demonstrate that the significance of nitrogen in nutrient–chlorophyll relationships in lakes probably reflects natural variability in phosphorus concentration, and not the fertilizing effect of nitrogen.

Résumé : Les écologistes utilisent fréquemment les relations statistiques pour inférer comment les variables sont véritablement reliées. Cependant, la méthode habituellement utilisée à cette fin (moindres carrés ordinaires (MCO)) est généralement déficiente à cause de l'importante variabilité naturelle de la plupart des variables écologiques. La variabilité naturelle des variables indépendantes peut artificiellement générer un patron empirique significatif même s'il n'existe aucune relation structurale, et peut également rendre indétectable une relation structurale véritable. Dans les relations multivariées, la variabilité naturelle dans une des variables indépendantes peut induire la signification statistique d'autres variables colinéaires même si ces dernières ne sont pas reliées de façon structurale. Nous proposons une nouvelle méthode simple, basée sur le principe des variables instrumentales, pour détecter et quantifier la variabilité naturelle des variables indépendantes et pour estimer les paramètres de la relation structurale. Nous appliquons cette méthode à deux exemples: (1) nous démontrons que le relation structurale entre la concentration d'adénosine triphosphate (biomasse planctonique totale) et la concentration de chlorophylle (biomasse autotrophe) ne varie pas selon la latitude dans l'océan antarctique en dépit d'une augmentation significative des pentes MCO vers le sud et (2) nous démontrons que la signification statistique de l'azote dans les relations entre éléments nutritifs et chlorophylle dans les lacs reflète probablement la variabilité naturelle des concentrations en phosphore, et non pas l'effet fertilisant de l'azote.

Introduction

Natural variability is a curse to ecologists. Experimental replications in the field are notoriously capricious, time-series records of population fluctuations may be indistinguishable from random numbers, and even apparently strong theoretical principles express themselves in loose empirical relationships. A large part of our effort is therefore directed to ferreting out, through extreme experimental manipulations or through brute force of sampling effort,

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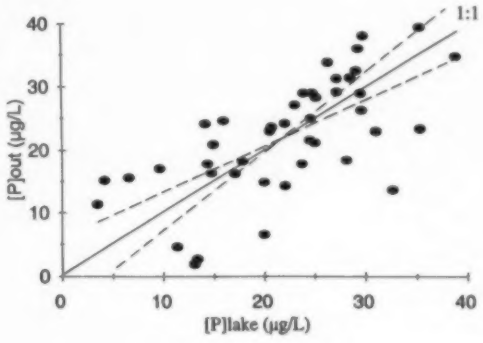
the underlying "true" or structural relationships driving ecosystem dynamics. In the search for this signal, however, little effort is directed toward the nature of the ecological noise that obscures it. Yet because natural variability is an inextricable part of ecological relationships, and because it may strongly influence our perception of those relationships, it must be addressed directly. We must learn to quantify and qualify natural variability.

Very few studies have attempted to look directly at unexplained variability and its influence on aquatic studies even though wide-ranging, noisy empirical relationships form some of the broadest generalizations in limnology. Law-like patterns such as the self-thinning rule for plants (Weller 1987), allometric relationships (Peters 1983; Calder 1984), nutrient response curves in lakes (Peters 1986), Taylor's power law (Taylor 1961), other mean–variance relationships (Downing 1979) and normalized size spectra of plankton communities (Sprules and Munawar 1986; Boudreau et al. 1991), as well as the widely cited large-scale production–biomass relationships are all important ecological regularities. Although their existence is not generally in dispute, the functional interpretation of empirical equations is more controversial largely because a relationship between ecological variables may have many plausible explanations that are often distiguished more by their elegance than by the merits of their predictive power (Peters 1991). Because empirical relationships are noisy, it is of little surprise that debates over the proper interpretation should arise.

Interpreting a quantitative ecological relationship functionally can mean several things. In its simplest form, it refers to whether or not a relationship exists between the variables, were we able to eliminate natural variability. In more complicated situations, it extracts additional information about the form of the relationship. For instance, it may be useful to know whether one variable varies in direct proportionality with the other (H_0: slope = 1 for a log–log relationship) or whether the parameters of an observed relationship are those predicted by theory. For example, allometricians were long concerned to know if metabolic rates characteristically scale as weight raised to the 2/3 or 3/4 power. Similarly, important claims have been made concerning the existence of an ecological scaling factor as evidenced by a steeper productivity–biomass relationship within ecologically similar organisms (Banse and Mosher 1980; Dickie et al. 1987). Unfortunately, the natural variability associated with ecological observations is often sufficiently large that such functional interpretation of empirically derived relationships is subject to serious shortcomings.

The whole question of natural variability in the functional interpretation of empirical relationships is essentially a statistical problem, albeit a difficult one. Most ecologists recognize that if a regression equation is applied in the same conditions under which the original data were collected, ordinary least-squares (OLS) regressions make the best prediction. However, prediction is not the only role given to regression models in ecology. More often than not, regression analysis is used in an attempt to interpret the underlying biological relationships. In this paper, we show that such functional interpretation derived from standard regression methods can be erroneous, particularly where "natural error variability" is large. We contend that the severity of this problem has been greatly underestimated and that this natural error variability, coupled with standard statistical methods, is likely (a) to reveal relationships where none exist, (b) to obscure existing relationships, or (c) to seriously bias the interpretation of observed relationships. After briefly reviewing a few of the more common regression techniques, we present a new and simple approach, based on auxiliary information in the form of instrumental variables, to detect the presence of significant effects of error variability and to estimate the model parameters of interest. In addition, the method allows the quantification and partitioning of this natural variability.

Fig. 1. Relationship between outflow and lake P concentrations in Lake Cromwell. The solid line is the 1:1 relationship; the two broken lines are OLS lines for $[P]_{out}$ versus $[P]_{lake}$ (shallow) and $[P]_{lake}$ versus $[P]_{out}$ (steep).



Example of the problem of interpretation

Suppose we wish to test the widely held assumption in mass-balance models of lake phosphorus that phosphorus concentrations in the lake and in the outflow are the same (Reckhow and Chapra 1983). If the assumption is correct, a scatter plot of the observations of lake P concentration ($[P]_{lake}$) versus outflow P concentration ($[P]_{out}$) in a single lake over time or among lakes should fall on or close to the 1:1 line, i.e., have a slope of unity and a null intercept. Data from Lake Cromwell, Quebec (Y.T. Prairie, unpublished data) appear to support the assumption (Fig. 1), but a standard regression line fitted to those points reveals that the observed relationship differs from expectation in that its slope (0.7) is significantly less than 1.0 ($p < 0.001$):

$$[1] \qquad [P]_{out} = 5.9 + 0.73\,[P]_{lake}, \qquad r^2 = 0.45$$

Furthermore, because the intercept is not significantly different from zero, the observed slope implies that lake outflow P concentrations are, on average, only about 75% of that in the lake. Several mechanisms could explain the discrepancy: sedimentation might be enhanced by aquatic macrophytes (Petticrew and Kalff 1992) or filter feeders (Morin et al. 1988) near or at the outflow, outflow waters

may reflect surface waters which are generally low in P (Hanna and Peters 1991), or zooplankton may affect the outflow of P by avoiding lake outlets (Ruttner 1953), or there could even be differential phosphine degassing in the littoral zone. Ecological papers often advance such a posteriori rationalizations (Peters 1991), providing intricate discussions about the possible meanings of these observations. Although this may be a useful way of generating new research hypotheses, it is legitimate only if relations like eq. [1] can be interpreted *functionally*. Unfortunately, such interpretations are defensible only if the regression method can aptly estimate the "structural" relationship (sensu Kendall and Stuart 1973) between the variables.

The folly of the proposed functional interpretations of eq. [1] can be illustrated easily by inverting the variables and repeating the regression analysis. The new equation

$$[2] \qquad [P]_{lake} = 8.5 + 0.61\ [P]_{out}, \qquad r^2 = 0.45$$

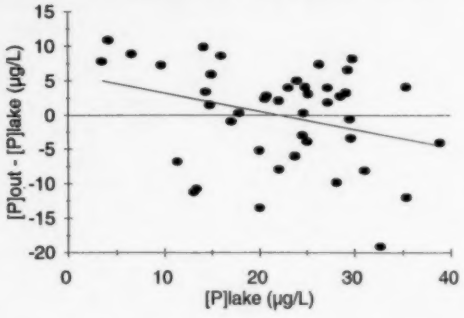
leads to an apparent paradox: we must reverse our interpretation because eq. [2] seems to show that lake P concentrations are significantly *lower* (by, on average, about 40%) than outflow concentrations. Clearly, the two interpretations are incompatible. There is no statistical reason to accept one over the other. All one can say with reasonable confidence is that the true value of the slope is probably somewhere between 0.6 and 1/0.6 (which equals 1.6), and this makes for a very poor test of the hypothesis H_1: $\beta \neq 1$.

The understandable desire of ecologists to interpret regression equations does not stop at an explanation of the regression parameters. It is not rare to attempt to further untangle the implications of a relationship. For example, we might examine whether the difference between lake and outflow P concentrations changes systematically with increasing lake [P]. In this case, regression analysis of $([P]_{out} - [P]_{lake})$ versus $[P]_{lake}$ reveals a significant negative relationship. Moreover, the corresponding scatter diagram (Fig. 2) clearly shows the existence of such a negative trend, albeit with a lot of noise. This trend is real. Nevertheless, its true meaning is not obvious: it is a significant empirical relationship that is built purely on natural error in the independent variable. Clearly, one requires a method that is both insensitive to axis reversal and produces functionally interpretable equation parameters.

Choice of methods

All statistical tests regarding parameter values are based on the values of their estimators and on their uncertainties, both of which are determined by the method used to fit the regression. For such tests to be valid, the method must provide unbiased estimates of all parameters. In other words, the data must meet the assumptions of the analysis. The assumptions for correctly interpreting regression statistics for lines fitted by OLS are well known: (1) the variables must be linearly related, (2) the residual variance must be homoscedastic, (3) the error term (residuals) must be random and normally distributed, and (4) the values of the independent variables must be fixed, or known without error. Techniques exist to evaluate whether or not the data violate assumptions 1, 2, and 3. Assumption 4 might seem easy to verify given that estimates of the precision of our measurements are usually available, but in most cases,

Fig. 2. Scatter diagram of the difference between outflow and lake P concentrations versus lake concentrations.



measurement errors are small relative to the whole range of the variable sampled, so this assumption is taken to be of little significance and is usually ignored. Although this may be appropriate in physics or chemistry, it can seriously mislead in ecology because error variability pervades our data and because we have wrongly equated natural error variability to measurement error.

Natural error variability

A phenomenon can exhibit high natural error variability even if measurement errors are negligible. For example, suppose we wish to examine how the zooplankton biomasses at two specific locations in the same lake covary through time. Our working hypothesis could be that they vary approximately synchronously because both respond to the same external environmental forcing. In this situation, the data to test this hypothesis are likely to exhibit low measurement error but high natural variability because of the patchy distribution of zooplankton (Pinel-Alloul et al. 1988). At one sampling, the zooplankton biomass at station A may be *truly* greater than at station B because there happened to be a swarm of animals there at that particular moment. The reverse may be true on the following sampling, even if the two stations respond to the external force in exactly the same way. Thus, the natural patchiness of zooplankton will induce variability in our estimates of abundance regardless of the precision of our measurements and this will bias any OLS regression analysis of these data. In this case, swarming constitutes a source of error because it is not variability we are interested in with regard to our initial hypothesis. However, it is "real" or natural variability and could be precisely the variability of interest in a different context (e.g., if one is looking for the grazing effects of fish schools). Thus this category of variability is termed "natural error variability". The reader should note that swarming or zooplankton patchiness is only one potential source of natural error variability; there may be many others, of known or unknown origin and magnitude. Ricker (1984) also discussed the two types of error variability encountered in ecological data.

Some solutions

Several authors have recommended the replacement of OLS methods with other slope estimators, such as the

ordinary major axis or the reduced major axis (also known as model II regression or geometric mean regression) (e.g., Ricker 1973; Jolicoeur 1975, 1990, 1991; Mark and Church 1977; Sokal and Rohlf 1981), when the observations are believed to be uncertain (due either to measurement errors or natural error variability). Jolicoeur (1991) discussed the merits and drawbacks of each. It is imperative to note, however, that the appropriateness of any of these slope estimators depends only and strongly on whether the data at hand conform to the very specific and restrictive assumptions each of these techniques makes about the error variances of the variables (McArdle 1988). For instance, the estimation of ordinary major axis makes the assumption that both the dependent and independent variables have identical natural error variabilities. This is likely appropriate if one wishes to regress, say, the length of the right arm against the length of the left arm. In most cases, however, and especially when the two variables of interest are conceptually very different, the appropriateness of this slope estimator for a particular data situation is a priori not knowable because the error variances include the inherently unmeasurable natural error variability component. Other problems exist with this particular technique (Jolicoeur 1990). Similarly, the popular geometric mean regression (GMR) technique makes an analogous assumption by which the two variables must have equal error variances when expressed in their reduced form (i.e., divided by their respective standard deviations). Clearly, the same limitations apply. Both of these techniques are therefore of little use to the empiricist. The advice often found in the ecological literature regarding the use of these alternative regression techniques (in particular, the model II or GMR regression, Ricker 1984) should be tempered with the realization that, without an estimate of the natural error variability component (not just measurement errors), these estimators are equally likely to be worse than they are to be better than the OLS estimates. The only solution is to find a simple and reliable method to estimate the structural relationship between the variables.

Structural relationship

An excellent summary of the mathematics of the structural relationship is given in Kendall and Stuart (1973). Briefly, the structural relationship expresses the exact linear relationship between two variables, the values of which may or may not be directly observable because of random fluctuations from natural error variability, from variability due to measurement errors, or from both. Depending on whether the true values of the variables are considered fixed or random variables themselves, the relationships are conventionally termed functional and structural, respectively (Kendall and Stuart 1973; Fuller 1987). However, because the estimation problem is applicable to both situations, the two epithets could be used here. Structural relationships are rarely estimated in ecology (Jensen 1986; McArdle 1988) and, for this reason, we offer a brief overview.

Given a true relationship

$$[3] \quad y = \alpha + \beta_{yx} x$$

where both x and y are subject to a random error such that

$$[4] \quad Y = y + \epsilon_y \quad \text{and} \quad X = x + \epsilon_x,$$

we seek to find a simple method to estimate β_{yx} from data. The variables X and Y are therefore values of x and y observed with random error ϵ_x and ϵ_y. Since the errors in x (ϵ_x) are presumed random (i.e., uncorrelated with x), we have for any sampled range of x

$$[5] \quad \text{var}(X) = \text{var}(x) + \text{var}(\epsilon_x)$$

and because errors in x or y do not affect the covariance between them ($\text{cov}(yx) = \text{cov}(YX)$), the OLS slope estimator is

$$[6] \quad E[\beta_{YX}] = \text{cov}(YX)/(\text{var}(x) + \text{var}(\epsilon_x))$$

whereas the true slope is

$$[7] \quad \beta_{yx} = \text{cov}(yx)/\text{var}(x).$$

Therefore, it can be seen that the OLS slope (β_{YX}) of Y on X will be biased toward zero relative to the true slope of the relationship (β_{yx}). The OLS slope (β_{YX}) is unbiased only when there is no error variability in x ($\text{var}(\epsilon_x) = 0$). This simple result implies that ordinary regression analysis may well reduce the OLS estimate of slope to statistical insignificance ($\beta_{YX} = 0$) when a structural relation actually exists ($\beta_{yx} \neq 0$). Combining eqs. [5] and [7] and making use of the equality

$$[8] \quad \text{cov}(yx) = \beta_{yx} \text{var}(x)$$

produces

$$[9] \quad E[\beta_{YX}] = \beta_{yx}[1 - \text{var}(\epsilon_x)/\text{var}(X)]$$

Equation [9] illustrates the "identifiability problem" in estimating the parameters of a structural relationship. Of the four parameters involved, two are estimable (β_{YX}, $\text{var}(X)$) from the data. Since β_{yx} is the parameter of primary interest, we must know the value of $\text{var}(\epsilon_x)$ to solve eq. [9]. More generally, β_{yx} can be estimated if at least one of the following estimates is available: $\text{var}(\epsilon_x)$, $\text{var}(\epsilon_y)$, or the reliability ratio $\text{var}(\epsilon_y)/\text{var}(\epsilon_x)$ (see Kendall and Stuart 1973, p. 397, for a lucid account).

There is a vast literature on models of this type in statistics (Madansky 1959; Cochran 1968, 1970; Haitovsky 1972; Seber 1977), the most recent and complete review of which is Fuller (1987). Such models are difficult in practice because reliable estimates require knowledge of both the measurement error (which can be obtained through proper replication) and the natural error variability (which cannot be readily estimated from data). Some possibilities exist. One may sample as wide a range in the predictor variable as possible so that $\text{var}(\epsilon_x)$ will be small relative to $\text{var}(X)$ in eq. [9], thereby making $\beta_{YX} \approx \beta_{yx}$ (although it is hard to know how "wide a range" is wide enough to ensure that $\text{var}(\epsilon_x)/\text{var}(X) \to 0$). Alternatively, one must use some auxiliary information. The following section describes how supplementary information in the form of "instrumental variables" can extract the natural variability component of the data and therefore provide us with reliable estimators of the parameters describing the structural relationship between the variables of interest (see Kuhry and Marcus 1977 for an early use of instrumental methods in biology).

Table 1. Sample statistics of the eight different data sets generated according to the simulations described in the text. var( ) and cov( ) are variance and covariance, respectively. OLS β is the slope fitted by ordinary least-squares. All data sets were generated using the same underlying structural model $y = 50 + 1x$.

var(X)	var(Y)	cov(XY)	OLS β
336.3	511	111.7	0.332
384.5	558	157.6	0.410
472.4	644	241.4	0.511
541.3	712	308	0.569
660.2	830	423.2	0.641
899.8	1065	656	0.729
1068.4	1232	821.6	0.769
1322	1485	1069.5	0.809

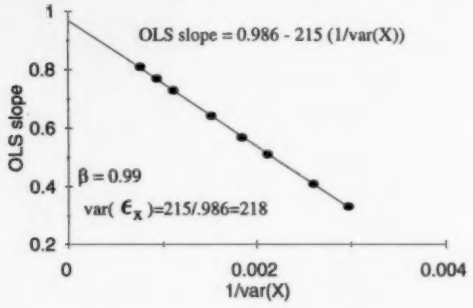
Instrumental variable methods

All methods involving instrumental variables are based on the principle that, in addition to measuring X and Y (alleged to have errors), we can measure a third variable (Z) that is correlated with the true values of x but not with the errors associated with it (ϵ_x). The nature of that third variable can be categorical (as in a classification) or numerical. Wald (1940) was the first to introduce an instrumental variable approach. His instrumental variable was a categorical variable distinguishing two groups of observations. A line passing through the centers of gravity of these two groups can be shown to estimate the true slope of the structural relationship. The more popular three-group method of Bartlett (1949) is an extension of Wald's estimator. Although both methods are in principle correct, some classic textbooks (Draper and Smith 1981; Sokal and Rohlf 1981) unfortunately state that observations can be ascribed to one of the two or three groups simply on the basis of the X values (i.e., by dividing the data in two or three groups according to the observed X values). This procedure is faulty, for it will generally induce a correlation between the observed X values and their error (ϵ_x), thereby invalidating one of the critical assumptions of the instrumental variable approach (Kendall and Stuart 1973; Fuller 1987). The remainder of this section will be devoted to the development of a new and simple instrumental variable method, called the slope–range method, and to a comparison of its efficiency relative to two other statistical techniques: the Reiersol (1950) estimator and a generalized least-squares approach to the problem (Fuller 1987).

Slope–range method

In the slope–range method, we use the instrumental variable principle in a different way. In a structural model, the X values have an error (ϵ_x) associated with them and this biases the OLS slope relative to β_{yx}. Equation [9] shows that the extent of the bias can be expressed as a function of the ratio of var(ϵ_x) to var(X). A given error variance will therefore affect the slope more if it is estimated over a small range of x. The slope–range method simply uses this property of OLS. Equation [9] can be re-arranged to show that the observed OLS slope (β_{YX}) is a linear function of the reciprocal of the observed var(X):

Fig. 3. Scatter diagram of OLS slopes versus 1/var(X) resulting from simulations of a single structural model in which the X variable spanned different ranges.



$$[10] \quad \beta_{YX} = \beta_{yx} - \beta_{yx}\,\mathrm{var}(\epsilon_x)\,(1/\mathrm{var}(X))$$

or

$$[11] \quad \beta_{YX} = \eta - \lambda\,(1/\mathrm{var}(X))$$

where

$$[12] \quad \eta = \beta_{yx} \quad \text{and} \quad \lambda = \beta_{yx}\mathrm{var}(\epsilon_x).$$

This result can be used when several instances of a relationship covering different ranges of the x variable (and hence X) are available. If nonnegligible error variability is present, plotting the observed OLS slopes against the reciprocal of the observed variances in X (technically, the variance of the sampled X over the limited ranges of x) will show a negative linear trend. The intercept of this relationship is an asymptotically unbiased estimator of the slope of the structural relationship, and the slope of the negative trend (λ) is an estimator of the product $\mathrm{var}(\epsilon_x)\cdot\beta_{yx}$. Because slopes and intercepts are generally correlated in a regression situation, the parameter var(ϵ_x) can be approximated using a Taylor expansion as

$$[13] \quad \hat{\mathrm{var}}(\epsilon_x) = E\left(\frac{\lambda}{\eta}\right) \approx \frac{\lambda}{\eta} - \frac{1}{\eta^2}\,\mathrm{cov}(\lambda\eta) + \frac{\lambda}{\eta^3}\,\mathrm{var}(\eta)$$

where cov($\lambda\eta$) is the covariance between λ and η obtained from the regression statistics of the β_{OLS}–1/var(X) relationship. However, in most of the situations we have encountered, simply using the first term of the approximation (λ/η) gave values within 10% of the full equation. Variances about the parameter estimates can be calculated as

$$[14] \quad \hat{\sigma}^2_{\beta_{yx}} = \sigma^2_\xi\left[1 + \frac{1}{n} + \frac{\bar{\psi}^2}{\Sigma(\psi_i - \bar{\psi})^2}\right]$$

and

$$[15] \quad \hat{\sigma}^2_{\mathrm{var}(\epsilon_x)} \approx \left(\frac{\lambda}{\eta}\right)^2\left(\frac{\mathrm{var}(\lambda)}{\lambda^2} + \frac{\mathrm{var}(\eta)}{\eta^2} - \frac{2\,\mathrm{cov}(\lambda\eta)}{\lambda\eta}\right)$$

where ψ is 1/var(X), σ^2_ξ and n are, respectively, the residual mean square and number of observations of the β_{OLS}–

Fig. 4. Relationship between log Chl *a* and log ATP concentrations for surface samples of the RACER study.

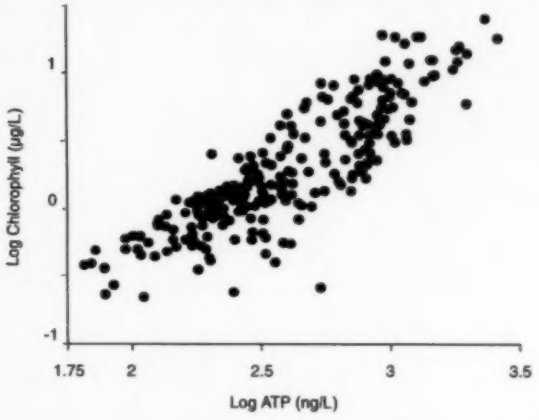
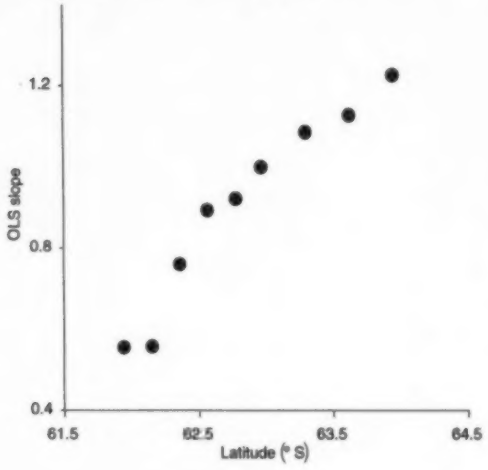


Fig. 5. Relationship between the OLS slope of the log Chl *a* – log ATP relationship and latitude.



1/var(X) regression, and var( ) and cov( ) denote variances and covariances. Examining eq. [14] shows that the uncertainty of the structural slope estimate will be smallest when both the number of groups (n) and the range covered by 1/var(X) in the slope–range diagram (the denominator of the last term of eq. [14]) are large. Similarly, if the number of observations within each of the groups is small, this will inevitably increase the scatter of the slope–range diagram (σ^2_ξ), thereby reducing the precision of the structural slope estimator. Nevertheless, the estimated structural slope remains unbiased and t-tests can then be applied as usual to assess statistical significance. As a rough practical guide, we suggest that about 10 groups be defined and that each group ideally contain >20 observations.

To illustrate the method, we developed a series of computer-generated data sets spanning different ranges of the X variable in which parameters of a structural relationship (eq. [2]) always remained constant. The sample statistics for these data sets are given in Table 1. All simulations were done on the SAS software package. We generated eight data sets of 500 observations, each with the same structural relationship ($y = 50 + 1x$) and the same normally distributed error variances (var(ϵ_x) = 225, var(ϵ_y) = 400) but spanning different ranges of x. We then calculated the OLS regression of Y on X for each data set and compiled the results in the slope–range diagram (Fig. 3). The observed OLS slopes were, as expected, negatively and linearly related to 1/var(X) and the slope–range method precisely estimated both the true slope of the structural relationship and the error variance we had imputed to the X variable (Fig. 3).

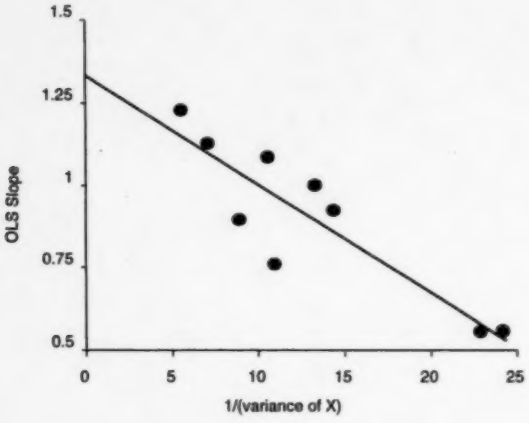
As with all line-fitting techniques, the slope–range method has assumptions. First, it assumes, like all other instrumental methods, that the errors (ϵ_x) in x are random, i.e., uncorrelated with the true values of x. Second, it also assumes that the grouping criterion is (a) uncorrelated with the errors in x and (b) effective in producing groups spanning different ranges in X. Last, it assumes that the error variance in X is also constant among groups. Except for the necessity that the grouping criterion produce subsets spanning different ranges, the other assumptions are common to all instrumental variable methods. In contrast with other grouping methods, however, it does not assume that the groups have different means (see Richardson and Wu 1970).

Some examples

Slope–range method using groups

The slope–range method can be used to examine and extract the natural variability component of many ecological relationships. We will illustrate the potential of the method by examining several of its properties using the relationship between ATP and chlorophyll concentrations in the sea. Whereas chlorophyll concentration is an indirect measure of algal biomass, the concentration of particulate ATP (<250 µm) can be used to estimate total microbial biomass (i.e., the biomass of both heterotrophic and autotrophic microplankton; Karl 1980). Karl et al. (1991) have shown the existence of a strong relationship between the two in the Southern Ocean as part of the Research on Antarctic Coastal Ecosystems Rates program (RACER: Huntley et al. 1991). The slope of the relationship using all data from the RACER study grid (25 000 km^2, 4-monthly cruises, 0–50 m depths) was not different from unity (log Chl *a* (µg/L) = 0.968 log ATP (ng/L) + 0.803, $r^2 = 0.69$, $n = 361$: Karl et al. 1991), suggesting that heterotrophic and autotrophic community size increased in unison over the very large community biomass range that was observed during the study. We asked whether the relationship (Fig. 4) holds equally in all antarctic marine habitats, in particular, whether the structural relationship between the phytoplanktonic primary producers and the secondary biomass they support changes between oligotrophic open-water stations to eutrophic coastal embayments. To test this hypothesis, the RACER study area (61°56.25′S to 64°14′S) was divided into nine latitudinal zones and standard OLS regression analyses were performed on the surface water data within each zone. The analysis revealed a clear latitudinal gradient in the ATP–chlorophyll relationship: the relationship

Fig. 6. Slope–range diagram for the log Chl *a* – log ATP relationship using the latitudinal zones as instrumental variable. With the natural error variability accounted for, the log Chl *a* – log ATP relationship has a structural slope of 1.33.



became progressively steeper at higher latitudes (Fig. 5). The slope varied from 0.56 in the Drake Passage to the north to 1.23 in the embayments of the Antarctic Peninsula to the south. A standard analysis of covariance indicated strong differences in the slope of the relationship among latitudinal groups ($F = 8.61$; df = 8, 242; $p < 0.0001$). Faced with such highly statistically significant results, we would surely feel justified in searching for an ecological explanation.

When we applied the slope–range method to the same data, however, another explanation emerged. A scatter plot of the relationship between the OLS slopes and the reciprocal of the variance of X for the nine latitudinal groups showed a significant linear relationship between the two that explains 80% of the variance in the OLS slopes (Fig. 6). This means that the statistical significance of the difference between latitudes was due largely or entirely to natural variability in ATP concentration among stations; there is no need to look further for differences in microbial community structure among latitudes on the basis of these data. The structural relationship common to all regions had a slope of 1.33 (SE = 0.122), which, being significantly greater than 1, indicates that at stations where autotrophic biomass was the greatest, the autotrophs formed a larger part of the community biomass than at poorer stations, contrary to what might have been concluded on the basis of OLS.

We have compared the results of the slope–range method for the ATP–chlorophyll example with the closely related method using generalized least-squares described in Fuller (1987). The two methods are related in the sense that they both use information derived from several different data sets. The latter method, although likely to be statistically superior, is computationally more complex even in the simplest case (with only two groups). In situations with a large number of groups (>20), the necessary computations may become prohibitive. For example, for 20 data groups,

Table 2. Comparison of the estimated parameters and their standard errors (SE) using the slope–range method and the generalized least-squares (GLS) approach. OLS statistics are also provided as reference.

Parameter estimated	Slope–range	GLS	OLS
β_0	−3.161	−3.194	−2.633
SE	0.319	0.238	0.116
β_1	1.328	1.336	1.124
SE	0.122	0.090	0.042
var(ϵ_x)	0.024	0.020	—
SE	0.0062	0.0059	—

the method requires the nonlinear fitting of a function with $20 \times 5 = 100$ variables. However, because this method is not well known in the natural sciences and that it could be particularly useful with a small number of groups, we provide a brief technical description of the technique in the Appendix. The interested reader should refer to Fuller (1987, section 4.2) for more details on the method. Using this technique, we obtained very similar estimators of the parameters of the structural relationship between log chlorophyll *a* (Chl *a*) and log ATP to those obtained from the slope–range method. The calculated standard errors were also very similar (Table 2). However, given the complexity of the approach and limited availability of programs able to perform this particular generalized least-squares application (e.g., not available in SAS), we suggest that the comparatively simple slope–range method provides a viable and attractive alternative. In addition, the method has the advantage that the statistics can be plotted (the slope–range diagram) and hence the effects of natural error variability visualized.

Slope–range method using a numerical instrumental variable

Like all instrumental variable methods, the additional information necessary to estimate the parameters of the structural relationship is contained within the grouping criterion itself. The grouping variable is the instrumental variable. Here we examine an even more effective use of the slope–range method by using a numerical (as opposed to categorical) instrumental variable to better constrain the regressor variable into groups spanning different ranges. The procedure is simple: given the usual variables X and Y, and a numerical instrumental variable Z known to be correlated with the true value of X but presumably not with its errors, one must produce several X–Y data sets by constraining the range of the Z variable to various degrees (i.e., by selecting observations forming different ranges in Z). These new data sets will necessarily span different ranges in the X variable. For each set, estimate the OLS slopes and 1/var(X) and plot the two in a slope–range diagram. If error variability in the regressor is at all important, a negative relationship should result. Conversely, if this error variability is nonexistent or negligible, the slope diagram will exhibit a null slope. We have optimized this procedure in a computer program that produces 100 data groups of different ranges in X (available from the first

Fig. 7. Slope–range diagram for the log Chl *a* – log TP relationship using log TN as instrumental variable.

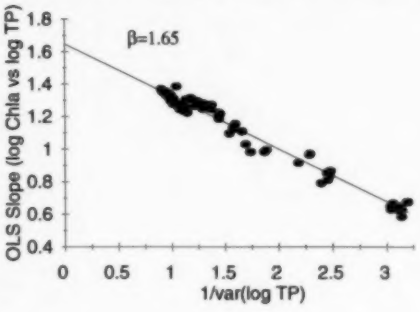
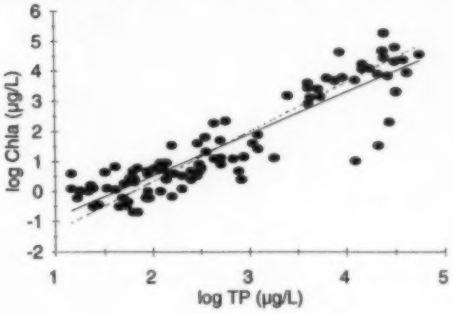


Fig. 8. Scatter diagram of the relationship between $\log_{10}$ Chl *a* and $\log_{10}$ TP. The solid line is the OLS fitted line; the broken line is the estimated structural relationship.



author upon request). It is essential to realize that constraining the *X* variable directly (as opposed to indirectly via the *Z* variable) will not work, for it will invariably induce a covariance between the observed *X* variable and its error. As a result, simulations show that directly restricted ranges of *X* will exhibit a constant OLS slope over any ranges of *X*, even in the presence of very large error variability in the regressor.

Note that, as with all instrumental variable methods, the criterion of independence between *Z* and the error in *X* can never be fully guaranteed and a certain measure of good sense must enter into the choice of the instrumental variable. Similarly, it is an implicit and not widely acknowledged assumption of *all* instrumental variable methods that the *Z* variable acts only as a surrogate of the *x* variable of interest, i.e., that it carries little explanatory power on *Y* other than that induced by its correlation with *x*. Our experience with the slope–range method on many complex simulated data sets shows that, although rare, some severe cases violating this assumption will produce highly atypical slope–range diagrams (e.g., exhibiting nonlinearity, heteroscedasticity, or even positive slope versus 1/var(*X*) relationships) with unreliable slope and error variance estimators. These symptoms are indicative of the inappropriateness of the instrumental variable used, and in such cases, we recommend its rejection. Nevertheless, the slope–range method has the advantage of allowing the detection of such difficult cases, something the other instrumental methods do not achieve because, unlike our method, the statistics cannot be visualized.

We applied the slope–range method using a numerical instrumental variable to the well-known relationship in aquatic ecology between phytoplankton biomass (as measured by Chl *a* concentration) and nutrients, especially total phosphorus (TP) (Dillon and Rigler 1974). There has been some debate in the literature as to the exact shape of this relationship, in particular how the yield of chlorophyll *a* per unit P increases with lake trophy. Statistically, this question amounts to estimating accurately the parameters of the structural relationship between log Chl *a* and log TP. We chose this example to illustrate the method because total nitrogen concentration (TN) is often measured together with chlorophyll and P and because log TP and log TN are well correlated (Downing and McCauley 1992). The variable TN thus fitted the criterion for a good instrumental variable. We used the data set of Prairie et al. (1989) and reexamined the structural relationship between chlorophyll and TP using the slope–range method with TN as a numerical instrumental variable. However, because of evident nonlinearity in the relationship at high TP values (their fig. 2), we confined our analysis to the linear portion of their graph (TP < 125). The simple OLS regression equation of chlorophyll on P is $\log_{10}$ Chl *a* = −2.27 + 1.39 $\log_{10}$ TP.

The slope–range diagram shows a strong linear and negative trend, thereby confirming the presence of substantial natural error variability in our TP values (Fig. 7). We found that the slope of the structural relationship between log Chl *a* and log TP was 1.65, much greater than usually acknowledged (Fig. 8). Most authors have reported slopes in the neighbourhood of 1.0 (0.8–1.3), although original slopes in the data of Sakamoto (1966) and Dillon and Rigler (1974) were higher (1.65 and 1.44, respectively). The estimated error variance in $\log_{10}$ TP is 0.2 (0.324/1.65). We performed three independent checks on the consistency of our estimated structural slope and error variance. First, we compared them with another method that uses a numerical instrumental variable (Reiersol 1950). The estimator β' was calculated simply as the ratio of the covariances (cov(*YZ*)/cov(*XZ*)) and, for this example, was equal to 1.59. Using eq. [9], the associated error variance in $\log_{10}$ TP is 0.14. Second, we estimated the structural relationship using the generalized least-squares approach (see the Appendix) using three data groups spanning different ranges in the TP variable and obtained estimates for the slope and error variance of 1.72 and 0.21, respectively. Finally, we also evaluated, using the slope–range method, the structural equation of the reverse relationship, namely of $\log_{10}$ TP as a function of $\log_{10}$ Chl *a*. In theory, one would expect the structural slope of the TP – Chl *a* relationships to be the reciprocal of that of the Chl *a* – TP relationships (i.e., 1/1.65 = 0.606). Our estimate using the slope–range method was, fortuitously enough, 0.61. The estimated error variance on the $\log_{10}$ Chl *a* values was 0.032, more than six times lower than that of $\log_{10}$ TP estimated with the same method. Given such a poor reliability ratio (0.032/0.2), it is little wonder that the OLS coefficients of Chl *a* – TP relationships are so biased. Although slightly more variable than in the Chl *a* versus

ATP example, the three methods gave consistently high slope estimates with an average error variance in $\log_{10}$ TP values of 0.18.

Further effects of error variability in the independent variables

Here we discuss two other side effects of natural error variability in the independent variables that are known in the statistical literature but that seem to have eluded the attention of most natural scientists. The first concerns the linearity of a relationship when the X variable is subject to natural error variability. Lindley (1947) has shown that even if there exists a perfectly linear relationship between the variables x and y and if x is observed with some error variability ($X = x + \epsilon_x$), the relationship between the measured values of X and Y will, in general, be nonlinear. The only situation in which linearity will be retained is when the distribution of the underlying x variable is normal (see discussions of this problem in Kendall and Stuart 1973, p. 430, and Fuller 1987, p. 75). For instance, if the distribution of x is uniform (a common occurrence), then the ensuing relationship between Y and X will be sigmoidal even if the error in x (ϵ_x) is itself normally distributed. There are many reports of a sigmoidal relationship in the aquatic ecology literature (e.g., TP–chlorophyll, McCauley et al. 1989; Prairie et al. 1989; all graphs supporting type III functional response curves), and the possibility that these are simply the result of natural error variability cannot be dismissed. Given the substantial natural error variability in TP–chlorophyll models, it is perhaps not surprising that McCauley et al. (1989) found significant log TP^2 and log TP^3 terms in their multiple regression model.

The second pitfall meriting wider recognition is that the effects of error variability on parameter estimation are even more pernicious in multiple regression. Not only can it bias OLS coefficients, but the presence of only one regressor with error can artifactually reveal other regressors to be significant even when these regressors are not subject to error variability themselves. Briefly, if a regressor variable X_1 is known with error and if a second regressor X_2 is correlated with the true values of X_1 (but not with its error), an ordinary multiple regression analysis will reveal both X_1 and X_2 to be significant, even if X_2 bears no real relationship with the dependent variable (Fuller 1987). Given that independent variables are very often collinear in ecological models, it is probable that some of the "significant" variables reported in the literature have no functional place in the equation (i.e., no "meaning"). This warning does not mean that the addition of significant (in the classical statistical sense) collinear variables does not increase predictive power. They clearly do. It does mean, however, that no interpretative meaning should be ascribed to these collinear variables, unless they can be shown to carry information relevant to the description of the dependent variable other than that induced by the error variability in the other regressor. Although the development of an extension of the slope–range method to estimate structural multiple regression equations will be the object of a separate paper, the foregoing discussion on nutrient–chlorophyll relationships leads us to question the validity of a multiple regression model of the type Chl $a = f$(TN,TP). In other words, is the "significant" influence of TN often reported in the literature (Smith 1982; Canfield 1983; Prairie et al. 1989) really expressing a fertilizing effect on algae, or is it significant because of natural error variability in TP and because TP and TN are well correlated? As a quick check, we tested whether the residuals of the *structural* log Chl a – log TP model were significantly related to log TN. To our surprise, the log TN variable was no longer significant ($p > 0.05$), suggesting that the significance of N in Chl $a = f$(TN,TP) models is likely artifactual. Furthermore, there has been some debate in the literature over the importance of N in these models because the additional information provided by the inclusion of N was small and because it could be substituted by nonlinear terms for P (e.g., 1/TP, Molot and Dillon 1991). Here again, our evaluation suggests that all these terms may well reflect the same debilitating influence of natural error variability in the independent variable.

Nature of natural error variability

The slope–range method provides information about a relationship in addition to a correct slope estimator: it provides us with an estimate of the natural variability associated with the variables we measured. These estimates can, in turn, be compared with other assessments of variability for that variable. For example, in the TP – Chl a case, the average calculated error variance in $\log_{10}$ TP (0.18) can be compared with the average temporal variability in that nutrient. It can be calculated from France and Peters (1992) that, for a mesotrophic lake (TP = 25 μg/L), the average annual temporal variance within a lake will be (on a $\log_{10}$ scale) 0.01.

What are the sources of natural error variability? We suggest that this variability can be partitioned into three components. First, measurement errors (analytical or human) may be a noninsignificant component of error variability in some cases. Second, the variable of concern may be subject to random temporal or spatial fluctuations unrelated to the phenomenon of interest. Last, this natural variability may simply reflect the slack in the biological mechanism underlying the relationship under study, i.e., how X can vary independently of Y. This is probably the greater component of natural error variability. In any case, given that natural variability is an integral part of our data and given its confounding effects in identifying ecological relationships, we suggest that this "noisy" side of nature should be given the consideration it rightly deserves.

Conclusions

A more valid description of nature can be gained in ecology by examining how variables are structurally or functionally related. Yet, the standard techniques used for this purpose are inadequate given the data ecologists generally face. Indeed, these techniques yield false interpretations because they ignore the confounding effects of natural variability. We advocate the estimation of the structural relationship for this purpose even if its predictive power is not optimal. The methods described herein, although imperfect, are a first step in this necessary direction.

Acknowledgements

We wish to thank Graham Bell for an insightful comment he made several years ago which eventually stimulated this research and which turned out to be essentially correct. We also thank Drs. David J. Currie, Pierre Legendre, Catherine Potvin, and Knut Seip as well as two anonymous reviewers for their valuable and insightful comments on the manuscript. Drs. David M. Karl and O. Holm-Hansen kindly made the RACER data available. The encouragement of Prof. Wayne Fuller of Iowa State University, and particularly his advice on generalized least-squares models, was pivotal in the final version of this manuscript. This work was supported by operating grants from the Natural Sciences and Engineering Research Council of Canada.

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Appendix

Generalized least-squares method

The operations involved in the estimation of the parameters of the structural model using the generalized least-squares approach consist of two steps. The first is the construction of a transformed data matrix derived from the sample variance–covariance matrices of the different data groups. The elements of this new data matrix are then fitted to a standard nonlinear regression program the parameters of which correspond to the parameters of the original model. Briefly, given a structural model of the form

$$Y = \beta_0 + \beta_1 x + \epsilon, \qquad X = x + u$$

which, we assume, applies to all different data groups (i.e., β_0, β_1, σ_{ee}, and σ_{uu} are constants). We can construct a variance–covariance matrix, m_i, for each group such that

$$m_i = \begin{matrix} \sigma_{YYi} & \sigma_{YXi} \\ \sigma_{XYi} & \sigma_{XXi} \end{matrix}$$

where σ_{YYi} and σ_{XXi} are variances and σ_{XYi} the covariance for data group i. If we have observations on the variables for k different data groups, the information vector s for this analysis is thus

$$s = [(\text{vech}(m_1))', (\text{vech}(m_2))', \ldots, (\text{vech}(m_k))', (\bar{Y}_1, \bar{X}_1), (\bar{Y}_2, \bar{X}_2), \ldots, (\bar{Y}_k, \bar{X}_k)]'$$

where $\text{vech}(m_i)'$ is the transposed half vector form of matrix m_i, i.e., $[\sigma_{YYi}, \sigma_{XYi}, \sigma_{XXi}]$.

The covariance matrix of the vector of covariances is estimated, for each data group, by

$$\hat{\Omega}_i = 2(n_i - 1)^{-1} \psi(m_i \otimes m_i)\psi'$$

where $\otimes$ is the Kronecker product and ψ is the unique matrix satisfying $\text{vech}\ m_i = \psi \text{vec}\ m_i$. Vec m_i and vech m_i are, respectively, the vector and half-vector forms of matrix m_i, and n_i is the number of observations in group i. For 2×2 variance-covariance matrices, ψ is

$$\psi = \begin{matrix} 1 & 0 & 0 & 0 \\ 0 & 0.5 & 0.5 & 0 \\ 0 & 0 & 0 & 1 \end{matrix}$$

Once the Ω matrix for each of the k data groups is found, we construct a block diagonal matrix $P'P$ such that

$$P'P = \text{blockdiag}(\hat{\Omega}_1^{-1}, \hat{\Omega}_2^{-1}, \ldots, \hat{\Omega}_k^{-1}, m_1^{-1}, m_2^{-1}, \ldots, m_k^{-1}).$$

We now need the matrix **P**, which can be found by Choleski decomposition of the block diagonal matrix above. It can be shown (see Fuller 1987) that the columns of this **P** matrix correspond to the "independent variables" of a nonlinear equation. The corresponding "dependent variable" is the matrix product **Ps** such that

$$\begin{aligned} \mathbf{Ps} = {} & (\beta_1\sigma_{xx1} + \sigma_{ee})\mathbf{P}_{.1} + (\beta_1\sigma_{xx1})\mathbf{P}_{.2} + (\sigma_{xx1} \\ & + \sigma_{uu})\mathbf{P}_{.3} + (\beta_1\sigma_{xx2} + \sigma_{ee})\mathbf{P}_{.4} + (\beta_1\sigma_{xx2})\mathbf{P}_{.5} \\ & + (\sigma_{xx2} + \sigma_{uu})\mathbf{P}_{.6} + \ldots + (\beta_1\sigma_{xxk} \\ & + \sigma_{ee})\mathbf{P}_{.(3k-2)} + (\beta_1\sigma_{xxk})\mathbf{P}_{.(3k-1)} + (\sigma_{xxk} \\ & + \sigma_{uu})\mathbf{P}_{.3k} + (\beta_0 + \beta_1\mu_{x1})\mathbf{P}_{.(3k+1)} \\ & + \mu_{x1}\,\mathbf{P}_{.(3k+2)} + (\beta_0 + \beta_1\mu_{x2})\mathbf{P}_{.(3k+3)} \\ & + \mu_{x2}\,\mathbf{P}_{.(3k+4)} + \ldots + (\beta_0 + \beta_1\mu_{xk})\mathbf{P}_{.(5k-1)} \\ & + \mu_{xk}\,\mathbf{P}_{.(5k)} \end{aligned}$$

where $\mathbf{P}_{.i}$ is the ith column of matrix **P**. The parameters of this model can then be estimated with any standard nonlinear regression programs. We used the procedure NLIN of SAS (Statistical Analysis System) throughout.

On the use of structured time-series to detect and test hypotheses about within-lakes relationships[1]

Yves T. Prairie and C. Tara Marshall

Abstract: Aquatic scientists using empirical relationships developed from point measurements or averages from different lakes often assume that these relationships also apply to individual lakes over time. However, this assumption is difficult to test because the extent of variation within a single system is generally much smaller and the relationship accordingly less defined than across a number of systems. We present a new method to extract empirical relationships from the internal structure of a time-series within a single lake. When we applied the method to an extreme simulation, we were able to recover accurately the parameters of the relationship in spite of the absence of any apparent relationship between the variables. When applied to empirical data for phosphorus and chlorophyll concentrations collected daily over one field season, the estimated structural relationship was nearly identical to that estimated from cross-sectional data even though the empirical trend appeared much shallower and very weak.

Résumé : Les scientifiques aquatiques utilisant les relations empiriques développées à partir de mesures ponctuelles ou de moyennes provenant de lacs différents font souvent la supposition implicite que ces relations peuvent être appliquées pour prédire le comportement d'un seul lac dans le temps. Or, cette supposition est difficile à vérifier parce que l'étendue des données à l'intérieur d'un seul système est généralement bien inférieure et la relation conséquemment moins bien définie qu'entre plusieurs systèmes. Nous présentons une nouvelle méthode d'estimation des relations empiriques basée sur la structure interne des séries chronologiques elles-mêmes. Nous avons appliqué la méthode à un exemple de simulation extrême et avons été capable de retrouver de façon précise les paramètres de la relation en dépit de l'absence manifeste d'aucune relation entre les variables. Lorsqu'appliquée à des données empiriques de concentrations de phosphore et de chlorophylle échantillonnées quotidiennement sur une seule saison de terrain à un seul site, la relation structurelle estimée était pratiquement identique à celle dérivée de moyennes provenant de plusieurs lacs et ce, même si le patron des données était beaucoup moins prononcé.

Introduction

Many important empirical relationships in limnology and fisheries science are derived from cross-sectional data, i.e., from point averages obtained for several lakes. These models often constitute the only tools to predict the expected value of state variables in new unsampled systems, but they also serve as backbones to many functional theories about ecosystems. Indeed, they form part of our current views on how the various compartments interact. However, because these relationships are generally developed from point measurements or averages for several lakes, one must be suspicious about whether the processes responsible for the large cross-sectional patterns also operate within a single system over time. In other words, we must ask if we can apply our cross-system relationships to explain the joint behaviour of the variables within a single lake over, say, the course of a season. In statistical terms, this is equivalent to asking whether the structural relationship (sensu Kendall and Stuart 1973) between the variables is the same among and within lakes.

The problems associated with appropriately estimating the parameters of a structural relationship between two variables have been discussed in greater detail elsewhere (Prairie et al. 1995). These problems essentially stem from the presence of substantial natural error variability in many ecological variables and the associated choice of fitting an algorithm (ordinary least-squares, geometric mean relationship, ordinary major axis, etc.). The difficulties are even more acute when trying to estimate them from a temporal series within a single system over time because the extent of total variation is usually so much smaller and the ensuing relationship much more poorly defined than

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across several systems. Yet testing such hypotheses is important because they may guide us in applying cross-sectional relationships to predict and explain the temporal behaviour of a single lake.

In this paper, we present a simple method for estimating the parameters of the structural relationship between two variables sampled in a single system over time. The method is a variant of that described in Prairie et al. (1995) but differs in that the additional information necessary to estimate the structural relationship is derived from the internal structure of the time-series themselves. After describing the principle of the method, we first torture-test it on an extreme simulation and then we apply it to empirical data of phosphorus and chlorophyll (Chl) obtained within Lake Memphremagog sampled at one station over a single growing season.

Methods

Statistical analysis

The method used here (called the slope–range method in Prairie et al. 1995) is based on the principle that when variables are subject to random fluctuations, ordinary least-squares (OLS) regression analysis will systematically bias the parameters of the structural relationship, i.e., the "true" relationship that would be revealed were the variables not subjected to random fluctuations. The extent of this bias will be greatest when the relationship is estimated on a data set covering only a small range in the independent variable (the X variable). More specifically, Prairie et al. (1995) have shown that the OLS slope should vary linearly and negatively with the reciprocal of the variance in X (i.e., $OLS_{slope} = \eta - \lambda\ (1/\text{var}[X])$) and that an unbiased estimator of the structural slope can be obtained as the intercept (η) of this OLS slope–1/var[X] relationship. Similarly, the variance of the random fluctuations in X (var[ϵ_x]) can be approximated as λ/η (see Prairie et al. 1995 for details). To use this method, however, one needs a third variable, called instrumental, to define groups of observations spanning different ranges (variances) in the X variable. Such third variables are not always available, but because data from a single system necessarily form a time-series and because time-series are often structured, it is possible to use this structure to circumvent the additional instrumental variable requirement. We begin by postulating a structural model of the form

$$[1] \quad y_t = \beta_0 + \beta_1 x_t$$

If the model holds through time, we also have

$$[2] \quad y_{t+h} = \beta_0 + \beta_1 x_{t+h}$$

and thus

$$[3] \quad (y_{t+h} - y_t) = \beta_1 (x_{t+h} - x_t)$$

showing that time-series differenced to the same degree (same h) in y and x will be structurally related with the same slope as the original variables. This is a useful trick because if the time-series are structured (i.e., data taken at short intervals are more similar than data further apart in time), it is likely that the pairs of differenced time-series for different values of h will span different ranges in the $x'_h = (x_{t+h} - x_t)$ variable, thereby satisfying a condition for the application of the slope–range method (see Prairie et al. 1995). Assuming, as one should without prior information, that both variables are subjected to random fluctuations such that we can only measure $X_t = x_t + \epsilon_x$ and $Y_t = y_t + \epsilon_y$ where ϵ_x and ϵ_y are the random fluctuations with variances var(ϵ_x) and var(ϵ_y) respectively, then we can construct several different pairs of differenced time-series (using different h values). For each pair, one calculates the OLS slopes and the variances in the new independent variables (X'). Plotting the slopes against the reciprocal of the X' variances should produce the expected negative linear trend. The statistics of this slope–1/var[X] relationship can then be used to estimate the parameters of the structural relationship: the intercept is an estimator of the structural slope and the natural error variance in X is approximated by λ/η. Technical details related to estimating the parameters and their uncertainties can be found in Prairie et al. (1995). It is important to note that the variance of the random errors associated with each value of the differenced time-series will be twice that of the original series due to the differencing operation.

Simulation data

We first tested the method described above using a simulated time-series of 100 data points for both x and y. The relation between the two variables was defined to be $y = x$, so the structural slope of this relationship is unity. We imposed a moderate degree of internal structure on the time-series by allowing the x variate to follow a one-dimensional Brownian trajectory (i.e., a random walk). Normal random fluctuations of specified variances (var(ϵ_y) and var(ϵ_x)) were then added to both variables to produce the observed time-series variables X_t and Y_t. We settled on particular values of var(ϵ_y) = 4900 and var(ϵ_x) = 2809 such that a simple scatter diagram of Y_t versus X_t would display visually no discernible pattern. We therefore chose the extreme situation where we know a structural relationship (of slope 1) exists between variables but where the natural variability is so large that there is no evidence of any relationship between them. The test of the method is whether it can detect and accurately quantify the underlying structural relationship.

Lake data

We applied the method on time-series of P and Chl concentrations within a single lake. We chose this particular example for three different reasons: (1) there already exist estimates of this structural relationship among lakes (Prairie et al. 1995), (2) it would be an important result to know whether P and Chl are related structurally in the same way within and among lakes because the among-lake regression is often used to prescribe within-lake restoration measures and because there is evidence that this often appears not to be the case (Sas 1989), and (3) one of us (C.T.M.) had sampled Lake Memphremagog intensively for the variables at one site for one field season and found only weak evidence of coupling over this time period.

The lake data were collected from the middle of the central basin of Lake Memphremagog, a long and narrow

Fig. 1. Time-series of the two simulated variables (Y is the solid line and X is the broken line) related as $y = x$ but confounded by random fluctuations.

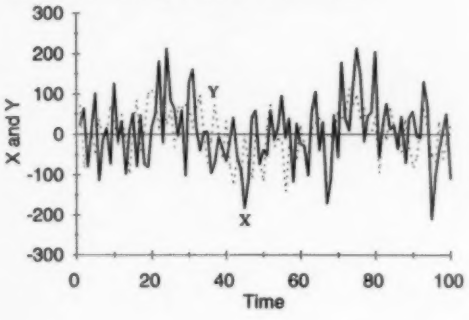


Fig. 2. Scatter diagram of Y versus X.

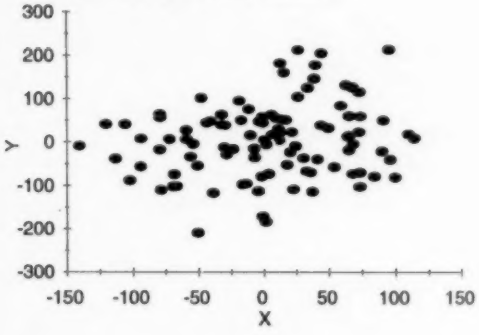


Fig. 3. Semivariogram of the X time-series.

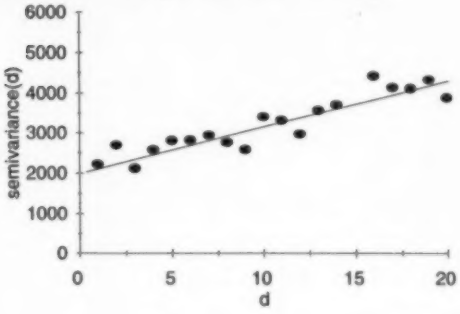
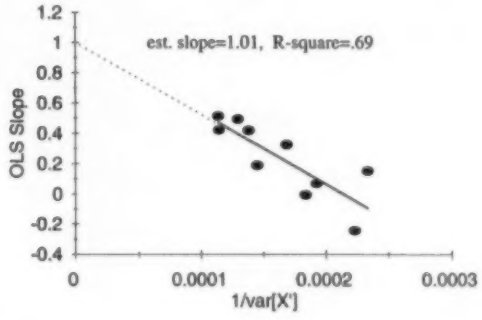


Fig. 4. Slope–range diagram relating the OLS slopes of the Y' on X' relationships as a function of 1/var(X'). Estimated structural slope (1.01) is read from the intercept.



mesotrophic lake on the Quebec–Vermont border. Duplicate volume-integrated samples of the photic zone (defined as twice the Secchi depth reading) were collected daily for 135 days in the summer of 1985. The concentration of total phosphorus (TP) was determined spectrophotometrically using the molybdenum-blue method following persulfate digestion and autoclaving (Menzel and Corwin 1965). Chl concentrations were determined using the trichromatic method after pigment extraction in 90% acetone (Wetzel and Likens 1991).

Results and discussion

Simulation results

The time-series of the two simulated variables, structurally related as $y = x$ but confounded by random fluctuations, are shown in Fig. 1. Although there appears to be some degree of association between the two variables, the corresponding scatter diagram displays only a very vague linear trend (Fig. 2, $R^2 = 0.03$). Ordinary regression analysis of these data revealed no statistically significant relationship, although the probability was borderline ($p = 0.07$), reflecting the large number of data points. We first examined whether the time-series displayed some internal structure by constructing a semivariogram of the X variable (see Robertson 1987). A semivariogram is basically just a plot of the variances among observations separated by a spatial or temporal distance d as a function of d itself. Structured time-series exhibit positive semivariograms near the origin showing that nearby (in time or space) values are more similar than distant ones. The semivariogram for the X time-series and the positive relationship (Fig. 3) clearly confirms the existence of some structure, making it possible to use our method. Thus, the data were differenced to various extents to produce 10 new pairs of time-series using differencing values (h) of 1, 2, 3, 4, 7, 10, 15, 20, 25, and 30. A greater number of small values of h were favoured because the internal structure of the time-series is strongest at small time scales. As expected, the new pairs exhibited different ranges in the X' variables, and the slopes (OLS) increased with increasing ranges producing a strong negative and linear slope–range diagram (Fig. 4). The estimated slope of the structural relationship is 1.01, almost exactly the value we had imposed on the data. However, the close similarity of the two slopes is probably fortuitous, for the standard error of the estimated structural slope is ±0.24 (see Prairie et al. 1995 for equations to calculate uncertainty of structural slope). Given the means of the two original time-series, we can calculate the structural equation of this relationship to be $Y = 2.2 + 1.01X$. The intercept (2.2) is not significantly different from zero. In contrast, the estimated structural slope is highly significant (t-test, $p = 0.001$). The standard error of the random fluctuations we had imputed on the x was 53 ($\sqrt{2810}$) and

Fig. 5. Time-series of TP and Chl concentrations measured daily at one site in Lake Memphremagog for 135 days in the summer of 1985.

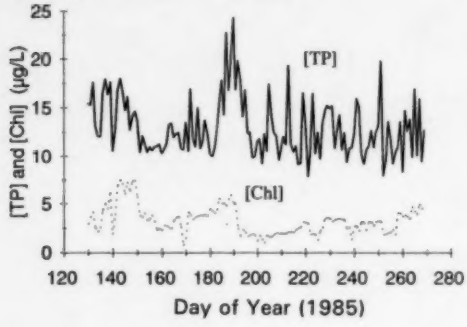
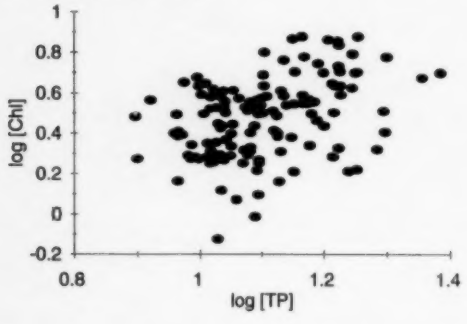


Fig. 6. Scatter diagram of log[Chl] as a function of log [TP] in Lake Memphremagog, summer 1985. Concentrations in µg/L.



that obtained from the analysis was somewhat larger at 69, although not significantly different (F-test on the variances, $p > 0.05$). Thus, the simulation results suggest that even in the extreme case that no significant correlation is observed in the raw data, the method was able to accurately recover the parameters of the structural relationship. We performed several other simulations (not shown) with less pathological conditions and there the method performed even better.

Application to the Lake Memphremagog data

Although some seasonality is apparent in the daily time-series of both TP and Chl for the summer of 1985 (Fig. 5), their synchrony is far from perfect and the resulting scatter diagram (Fig. 6) provides little evidence for the sort of strong coupling often reported in the literature (e.g., Dillon and Rigler 1974; Smith 1982). Although statistically significant, P only explained 11% of the variation in Chl. More importantly, the OLS slope of this relationship is only 0.75 (±0.15), lower than most reported slopes (except for very low or very high total nitrogen (TN):TP ratio systems, Prairie et al. 1989) and much lower than the structural slope estimated by various instrumental variable methods examined by Prairie et al. (1995), which varied between 1.59 and 1.72. The relevant question to ask is

Fig. 7. Semivariogram of log [TP] time-series.

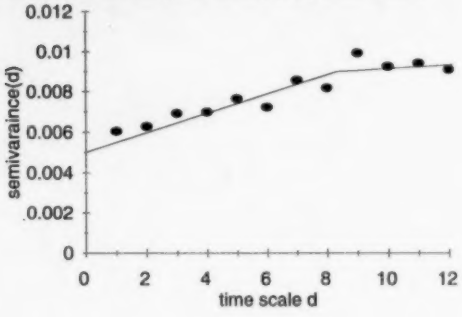
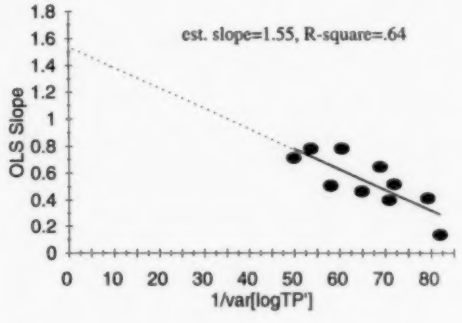


Fig. 8. Slope–range diagram of log [Chl] versus log [TP]. Estimated structural slope (1.55) is read from the intercept.



whether the fluctuations in Chl in Lake Memphremagog are structurally related to changes in P concentrations in the same way as that derived from many systems or whether the relationship is structurally different.

Before applying the differencing variant of the slope–range method, we examined whether the P time-series was temporally structured, again using a semivariogram for the $\log_{10}$[TP] values. We used logs because the TP–Chl relationship is usually best described on log values of both variables. There is a clear temporal structure (positive trend) up to a time scale of about 10 days (Fig. 7), above which the semivariance appears to stabilize and is therefore no longer useful in producing time-series with different ranges. For this reason, we constructed a total of 10 pairs of differenced time-series (h = 1–10 days), and the calculated OLS slopes between $\log_{10}$Chl versus $\log_{10}$[TP] were plotted against the reciprocal of the variance in $\log_{10}$[TP]′ (the prime refers to the differenced time-series). We obtained, as expected, a strong negative and linear trend (Fig. 8). More surprising, however, was that our estimated structural slope (1.55) is very close to that obtained in the cross-sectional data sets. This strongly supports the notion that, even within the short time scale of one field season and within a single station within a single lake, P and Chl are related in the same way as the cross-sectional relationships predicted. In fact, because these results are derived from differenced time-series using a *maximum* of 10 days

of differencing, our analysis implies that even daily changes in P concentration have consequences on the fluctuations of the Chl concentration. Standard regression analysis techniques are simply not powerful enough to detect them. The difference between the OLS slope (0.75) and the structural slope (1.55) stems from the relatively large natural error variability present in the P data. According to the slope–range method, the natural error variance in $\log_{10}$[TP] is (0.0153/1.55)/2) = 0.005, which is over half of the total variance in $\log_{10}$[TP] observed over the entire season. In the original metric, this natural error variance corresponds to a coefficient of variation of about 16%. Since uncertainty in X depresses the OLS slope, it is little wonder that the OLS slope is so biased downward in this case.

Our results can be corroborated independently. Reexamining the semivariogram (Fig. 7), we can see that although the variance among measurements taken at time intervals d increases with d, this variance is not nil when the trend is extrapolated to the origin (Fig. 7). In fact, this so-called nugget variance (see Robertson 1987) provides an independent estimate of the instantaneous natural error variability of the $\log_{10}$[TP] variable. In this case, the nugget variance is 0.0054, which is virtually identical to that obtained from our slope–range analysis (0.0050). The same observation can be made with regard to the simulation example above. Thus, combining the two statistical techniques (semivariograms and the slope–range method) provides a very powerful means of establishing the parameters of the structural equation linking the two variables of interest.

In summary, we have presented a simple variant of a structural relationship estimation method that relies on the internal structure of time-series to extract the error components and parameters of the structural relationship. The method appears to work in the extreme simulation and in the empirical example we examined, particularly when combined with a semivariogram analysis. The implication of this result is that many relationships deemed inapplicable or insignificant in particular systems should be reexamined.

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Longitudinal and seasonal development of planktonic chlorophyll *a* in the Rideau River, Ontario

B.K. Basu and F.R. Pick

Abstract: Planktonic chlorophyll *a* (chl-*a*) concentrations in the Rideau River, Ontario showed longitudinal and seasonal variation and ranged from 2 to 19 $\mu g \cdot L^{-1}$. Chlorophyll *a* concentrations in the river were not simply a reflection of the concentrations in the headwaters. On movement from the lentic headwaters into the lotic river waters there was usually a significant decrease in chl-*a* concentration. Downstream there were reaches of net increase in chl-*a* (sources), reaches of no change in concentration, and reaches of net decrease (sinks). Increases in concentration only occurred over reaches with retention times of 72 h or longer. No increases in chl-*a* concentration occurred over a reach with a retention time less than 50 h. Chlorophyll *a* concentration was not significantly correlated with discharge. Chlorophyll *a* concentration was positively related to total phosphorus concentration ($R^2 = 0.15$, $p = 0.016$). About 50% of the variation in chl-*a* concentration could be accounted for by a combination of total phosphorus, nitrate, and soluble reactive phosphorus concentrations.

Résumé : Les concentrations de chlorophylle *a* plantonique dans la rivière Rideau, en Ontario, accusent une variation longitudinale et saisonnière; elles oscillent entre 2 et 19 µg/L. Les concentations de chlorophylle *a* dans ce cours d'eau ne reflétaient pas simplement les concentrations dans les eaux du cours supérieur. Des eaux lénitiques d'amont aux eaux lotiques situées plus bas, on constate habituellement une baisse considérable de la concentration de chlorophylle *a*. En aval, sur certains passages, on a mesuré des augmentations marquées de la concentration (sources), sur d'autres passages, aucun changement n'a été constaté, et, dans d'autres secteurs, une diminution nette a été enregistrée (drains). Les augmentations ont été notées uniquement sur les tronçons où la période de rétention est de 72 heures ou supérieure. Aucune hausse de la concentration de chlorophylle *a* n'a été constatée sur des tronçons où la période de rétention est inférieure à 50 heures. Aucune corrélation significative n'a pu être établie entre le débit et la concentration de chlorophylle *a*. On a toutefois établi que la concentration de chlorophylle *a* est directement proportionnelle à la concentration de phosphore total ($R^2 = 0{,}15$, $p = 0{,}016$). Environ 50% de la variation de la concentration de chlorophylle *a* peut être attribuée aux concentations combinées de phosphore total, de nitrate et de phosphore soluble réactif.
[Traduit par la Rédaction]

Introduction

Phytoplankton dynamics have been studied extensively in lentic freshwaters (lakes and reservoirs), yet comparatively little research has focused on lotic waters (rivers) (Reynolds 1988; Murakami et al. 1992). As a result, the factors that regulate the spatial and seasonal development of suspended algae in rivers are poorly known. Previous work on rivers has demonstrated the regular development of extensive algal biomass in medium to large rivers. Many European lowland rivers can develop chlorophyll *a* (chl-*a*) concentrations over 100 $\mu g \cdot L^{-1}$ (Lack et al. 1978, Jones 1984; Moss et al. 1984; Jones and Barrington 1985; Descy 1987, de Ruyter van Steveninck et al. 1992; Kohler 1993). Chlorophyll *a* levels over 100 $\mu g \cdot L^{-1}$ have also been observed in large North American rivers (Baker and Baker 1979, Soballe and Bachman 1984). In contrast, some large rivers have low concentrations of chl-*a*. Lewis (1988) observed chl-*a* concentrations below 0.5 $\mu g \cdot L^{-1}$ in the Orinoco river. Furthermore, algal biomass levels in rivers exhibit extensive longitudinal heterogeneity (Soballe and Bachman 1984; Chessman 1985; Cole et al. 1992).

The suspended algae of large rivers consist primarily of algae originating from two sources (Hynes 1972): (*i*) lentic algae, derived from standing waters in which there is an export of planktonic species into the draining tributaries (Talling and Rzoska 1967; Reynolds 1988; Moss et al. 1989) and (*ii*) potamoplankton: algae capable of in situ reproduction within flowing water (Reynolds 1988;

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Murakami et al. 1992). In large rivers, algae of benthic origin, although present, represent a smaller fraction of total suspended algae (Lack 1971). True river phytoplankton or potamoplankton attains significant biomass only in large rivers where residence times and low flow rates allow planktonic organisms sufficient time for growth and reproduction (Margalef 1960; Lack 1971; Jones and Barrington 1985; de Ruyter van Steveninck et al. 1990*a*; Kohler 1993).

Large rivers in which potamoplankton develops are characterized by (*i*) water residence times ranging from days to weeks, which vary with discharge; (*ii*) continuous turbulent mixing; and (*iii*) an abundant nutrient supply due to an extensive association with their watersheds (Moss et al. 1989). These characteristics may affect potamoplankton development as follows.

Downstream advection in rivers may limit planktonic development by removing organisms faster than they can reproduce (Reynolds 1988; Pace et al. 1992). Within individual rivers potamoplankton biomass has been inversely correlated with the river's discharge (Jones 1984; Jones and Barrington 1985; Descy 1987; Reynolds 1988). Within a river, discharge itself is negatively correlated with water residence time. As Reynolds (1988) outlines, if potamoplankton biomass is to be maintained or increase within a large river, there must be reaches within the river which have long enough residence times so that in situ reproduction counteracts advective loss.

Turbulent mixing is beneficial to algal development because it can eliminate nutrient depleted water pockets adjacent to algal cells (Reynolds 1988). Turbulent mixing also may be detrimental to algal development by carrying phytoplankton below the photic zone (Cole et al. 1992). In deep sections of rivers, when the depth of mixing is greater than the depth of the photic zone, it is possible that the average irradiance to which algal cells are exposed is near or below the threshold for net growth (Cole et al. 1992; Lewis 1988). In shallower sections, with reduced mixing depths, the average irradiance may be sufficient to allow for net positive growth (Cole et al. 1992).

Increased nutrient concentrations should benefit potamoplankton development. However, Soballe and Bachman (1984) studying the Des Moines River and Sabater and Munoz (1990) working on the River Ebro have shown potamoplankton biomass to be less dependent on nutrient concentration than lentic phytoplankton biomass. Soballe and Kimmel (1987) using the NES (National Eutrophication Survey) and NASQUAN (National Stream Water Quality Accounting Network) data bases concluded that algal abundance in U.S. rivers was less predictable (lower R^2) on the basis of nutrient supply than in natural lakes.

The present study examined the longitudinal and seasonal development of suspended algae within a large Ontario river (Rideau River) of relatively high nutrient status (approximately 30 $\mu g \cdot L^{-1}$ of total phosphorus). Within the Rideau River, algae of benthic origin contribute less than 10% to total suspended algal biomass (J.R. Yang, University of Ottawa, Department of Biology, personal communication). By sampling seven sites on the river we examined the longitudinal differences in algal biomass over a 6-month period from May to October 1993. In addition, three reaches of varying water retention times were defined between sites in the river and the inflow algal biomass and outflow algal biomass of each reach measured to identify reaches of net algal development.

Firstly, we considered the hypothesis that potamoplankton biomass as measured by chl-*a* concentration gradually increases downstream. As the water moves downstream, the "time for development" increases and it is expected that algal biomass will increase. This gradual increase has been previously observed by Greenberg (1964), Capblancq and Descamps (1978), Holmes and Whitton (1981), and Moss et al. (1984). Secondly, the significance of factors that potentially regulate algal biomass in rivers was evaluated. These factors included temperature, nutrient concentration, discharge, and retention time. In short residence systems, abiotic factors such as river discharge or temperature may control algal biomass more than nutrient concentrations (Soballe and Kimmell 1987). Finally, we considered the hypothesis that algal biomass will increase through a river reach only when the retention time of the reach is sufficiently long that growth and reproduction exceed advective loss (Reynolds 1988). We anticipated changes in biomass over the reaches with longer retention times and no change over the reaches with shorter retention times.

Methods

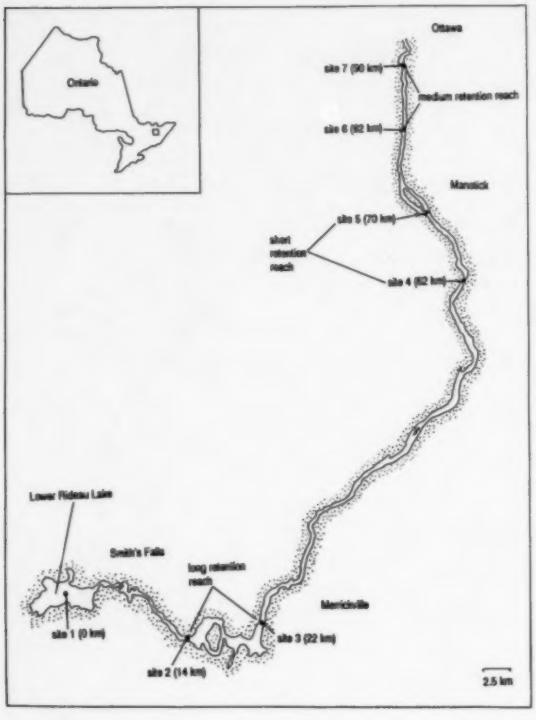
Study area

The Rideau River is located in southeastern Ontario. From its headwaters in Lower Rideau Lake, it flows in a northeasterly direction for 110 km before joining the Ottawa River at Ottawa (45°27′N, 75°42′W) (Fig. 1). The geology of the region consists of a mixture of sedimentary and Precambrian bedrock with large areas of silty clay and sandy loam (Davidson 1990). The watershed area of the Rideau at Ottawa is 3830 km^2 and the average annual discharge is 38.9 $m^3 \cdot s^{-1}$ (Water Survey of Canada 1990). Being lake fed, discharge does not change appreciably along the Rideau's length and there are no major tributaries within the section studied. Approximately 70% of the watershed area is agricultural land, the remainder is either urban or forested (Davidson 1990).

Seven sites (numbered 1–7) were sampled along the Rideau River. Site 1 was located in the headwaters, Lower Rideau Lake, while sites 2–7 were located within the river proper (Fig. 1). In addition, three reaches of varying water retention time were defined between sampling sites. The reaches were termed long, medium and short retention. The long retention reach was located between site 2 and site 3. The medium retention reach was located between site 6 and site 7. The short retention reach was located between site 4 and site 5 (Fig. 1). Each of these three reaches was 8 km in length.

The volumes of the reaches were determined by multiplying the surface area of each reach (determined by planimetry) by its average depth. Average depth was determined using a depth sounder (LCR 400ID, Marine Information Systems, Alabama) and available bathymetric maps. The average depths of the reaches with long retention, medium retention, and short retention were 2, 5, and 3 m,

Fig. 1. Location of sampling sites and defined river reaches of varying retention time along the Rideau River, Ontario. Distances refer to the number of kilometres downstream from the sampling site in Lower Rideau Lake (site 1).



respectively. Discharge values were obtained from the Water Survey of Canada, which maintains a continuous gauging site at Ottawa. Discharge was calculated as the average of the daily discharge for the period 7 d prior to and including the sampling dates in a manner similar to Pace et al. (1992). Theoretical retention times of the reaches were determined by dividing reach volume by discharge (Soballe and Threlkeld 1985).

Field sampling

At each site water samples were taken using a 4-m vertically integrated tube across a bank to bank transect. Samples were taken at the one third, one half, and two thirds distances across (Shaw 1987). It was assumed that the water column was vertically homogenous as has been shown for other rivers of similar size and discharge (Rosemarin 1975; Capblancq and Descamps 1978; Baker and Baker 1979; Pace et al. 1992; but cf. Pieterse et al. 1986). Furthermore, depth profiles of temperature obtained in early August indicated no vertical stratification. At each site, six 2-L samples (two at each distance across) were taken for algal chl-*a* and three 300-mL samples (one at each distance across) were taken for total phosphorus (TP), soluble reactive phosphorus (SRP), and nitrite + nitrate (NO_2 + NO_3) concentration determinations. Surface water temperature was measured with a mercury thermometer. Samples were stored in Nalgene™ plastic bottles and kept cool and dark during transport to Ottawa. Sampling was conducted once a month from May to October.

Laboratory analysis

Water samples were filtered through Whatman™ GF/F filters for chl-*a* analysis. Chlorophyll *a* was extracted using DMSO and acetone (Burnison 1980) and concentrations (uncorrected for phaeopigments) were calculated using the equations of Jeffrey and Humphrey (1975).

Chemical analysis was performed at the Regional Municipality of Ottawa–Carleton (RMOC) Surface Water Quality Laboratories using a SKALAR autoanalyzer. TP concentration was determined by acid digestion to orthophosphorous followed by reaction with ammonium molybdate and ascorbic acid (RMOC 1993). SRP concentration was determined by reacting ortho-phosphorous with ammonium molybdate and potassium antimony tartrate followed by reduction with ascorbic acid (RMOC 1993). Nitrite + nitrate concentration was determined by reducing nitrate to nitrite, diazotizing the nitrite with sulfanilimide, and coupling with ethylenediamine dihydrochloride (RMOC 1993).

Statistical analysis

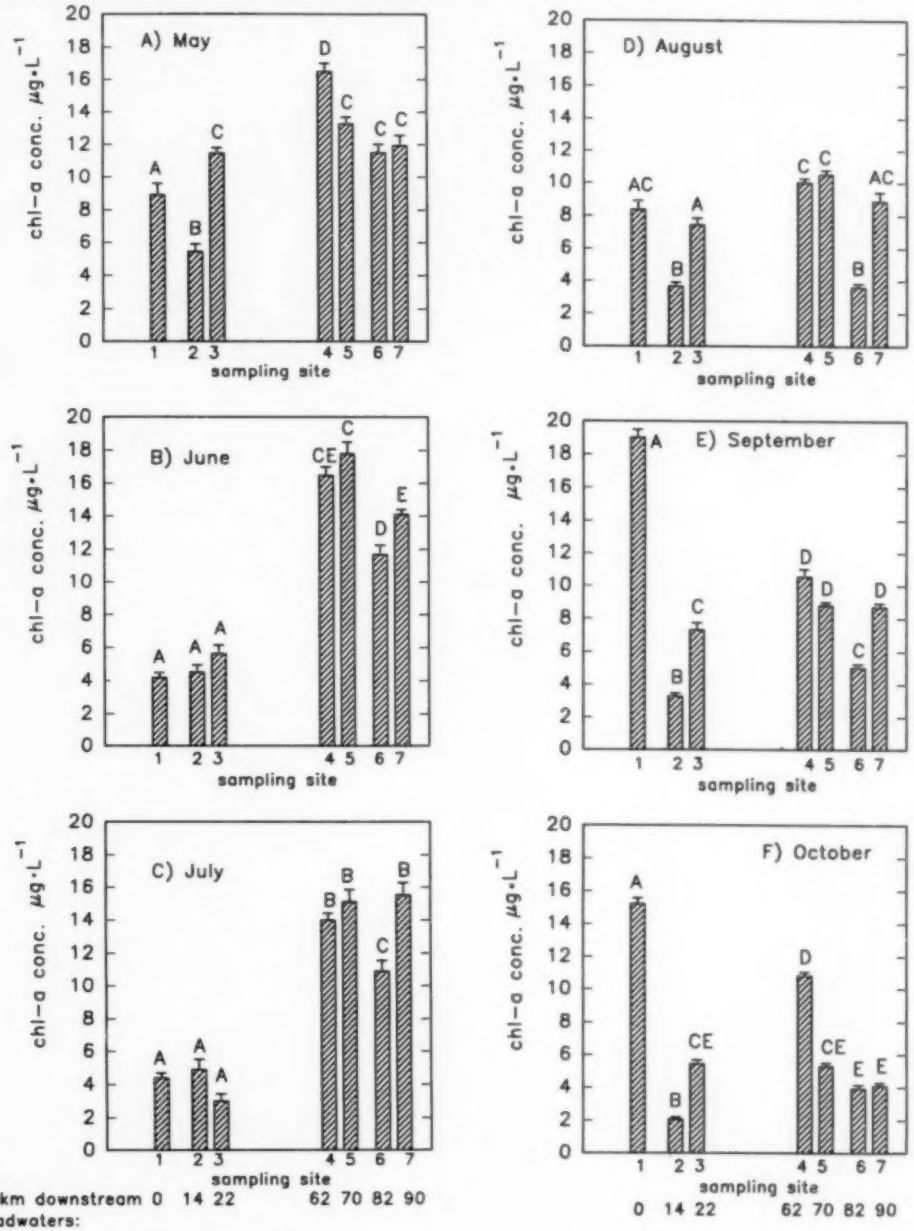
Statistical Analysis was performed using either SAS (SAS Institute Inc., Cary N.C.) or Sigmastat (Jandel Scientific, San Rafael, Calif.) statistical software. Parametric tests performed included two-way model I ANOVA, linear regression, multiple linear regression, and Tukey multiple comparisons. Where appropriate, type III sum of squares was used in interpretation. All parametric tests satisfied the assumptions of normality (Wilkes–Shapiro test) and homoscedasticity (Levene's test) following any necessary data transformation. Nonparametric two-way ANOVA and Tukey-type multiple comparisons (Zar 1984) were used when data could not be transformed to satisfy normality and homoscedasticity assumptions. The significance value for all statistical tests was 5% ($p = 0.05$).

Results

Chlorophyll *a* concentration: seasonal and spatial trends

There was considerable variation in chl-*a* concentrations both spatially and seasonally within the section of the Rideau River examined. Following data transformation parametric two-way ANOVA revealed significant differences in chl-*a* concentration among sites and among months (overall ANOVA, $p < 0.0001$, $F = 114.4$; for site, $p < 0.0001$, $F = 257.6$; for month, $p < 0.0001$, $F = 159.1$). A posteriori Tukey multiple comparisons revealed several trends with respect to chl-*a* among sites. Chlorophyll *a* concentrations decreased significantly from the headwater site (site 1) to the first river site (site 2) in the months of May, August, September, and October (Fig. 2). The largest decrease occurred in September (from 19 $\mu g \cdot L^{-1}$ to 3 $\mu g \cdot L^{-1}$). Following this initial decrease, chl-*a* concentration significantly increased from site 2 to site 3 in these months. From site 3 to site 4 a significant increase in chl-*a* concentration occurred in all months (Fig. 2). At

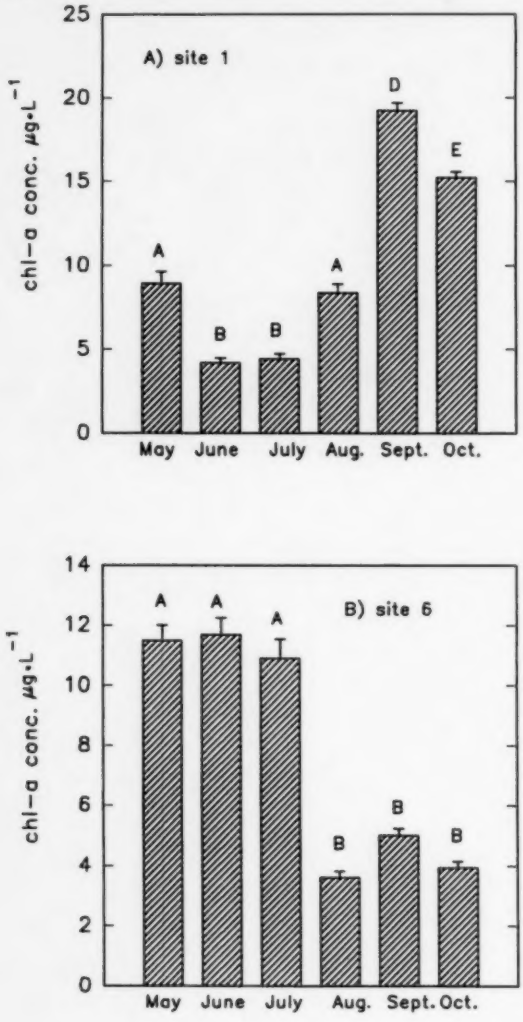
Fig. 2. Concentration of chlorophyll *a* in the Rideau River during (A) May, (B) June, (C) July, (D) August, (E) September, and (F) October 1993. Sites are those identified in Fig. 1. Plotted are means ± SE, $n = 6$. Means with same letter are not significantly different at 5%.



site 4 at least 10 $\mu g \cdot L^{-1}$ of chl-*a* was always observed. There was never an increase in chl-*a* concentration from site 4 to site 5 (Fig. 2). From site 5 to site 6 a significant decrease in chl-*a* concentration occurred in all months except May and October. From site 6 to site 7 significant increases in chl-*a* concentration occurred in all months except May and October (Fig. 2).

To summarize, the longitudinal trends that predominated over most months were a decrease in chl-*a* concentration from site 1 to site 2, an increase from site 2 to

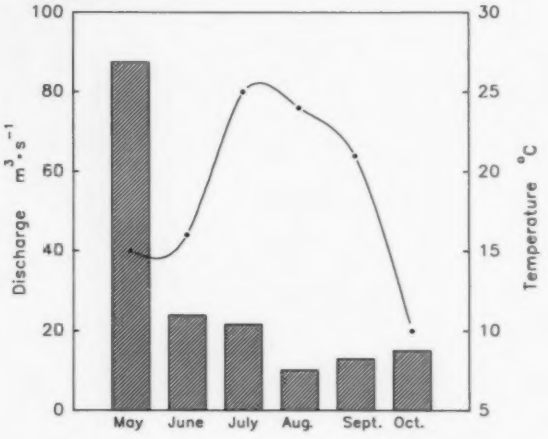
Fig. 3. Seasonal variation in chlorophyll *a* at site 1 (A) and site 6 (B) on the Rideau River in 1993. Plotted are means ± SE, $n = 6$. Means with same letter are not significantly different at 5%.



site 3, an increase from site 3 to site 4, no change from site 4 to site 5, a decrease from site 5 to site 6 and an increase from site 6 to site 7.

Tukey multiple comparisons revealed two seasonal trends in chl-*a* concentration among the river sites. Sites 1 and 2 exhibited a high concentration in May (between 9 and 11 $\mu g \cdot L^{-1}$ of chl-*a*) followed by a significant decrease in June and July (between 4 and 6 $\mu g \cdot L^{-1}$ of chl-*a*). This was followed by a significant increase from July to August and September. From September to October there was a significant decrease in chl-*a* concentration. (Figs. 2 and 3A). Site 2 exhibited no seasonal trend and chl-*a* biomass was always low (range from 2.05 to 5.43 $\mu g \cdot L^{-1}$, all months 4.26 ± 0.31 $\mu g \cdot L^{-1}$ (mean ± SE), $n = 34$). At the

Fig. 4. Seasonal pattern of discharge (bars) and temperature (line) for the Rideau River during sampling period 1993. Discharge for each month is the mean discharge for the period 7 d prior to and including sampling dates.



downstream sites (sites 4, 5, 6, and 7) a high concentration was observed from May until July (all sites 14.1 ± 0.03 $\mu g \cdot L^{-1}$, $n = 71$). This was followed by a significant decrease in August (all sites 6.3 ± 0.41 $\mu g \cdot L^{-1}$, $n = 24$) after which concentration did not change significantly (Fig. 3B).

Discharge and water temperature

The discharge hydrograph for the Rideau River is typical of that for other temperate Ontario rivers (Water Survey of Canada 1990). A high spring discharge of approximately 90 $m^3 \cdot s^{-1}$ in May was followed by lower discharges in the summer months. The lowest discharge occurred in August (10.0 $m^3 \cdot s^{-1}$) (Fig. 4). River water temperature also exhibited a distinct seasonal pattern with a low of 10°C in October and a high of 25°C in July (Fig. 4). In any one month, there were no significant longitudinal differences in temperature along the river.

Nutrient concentrations seasonal and spatial trends

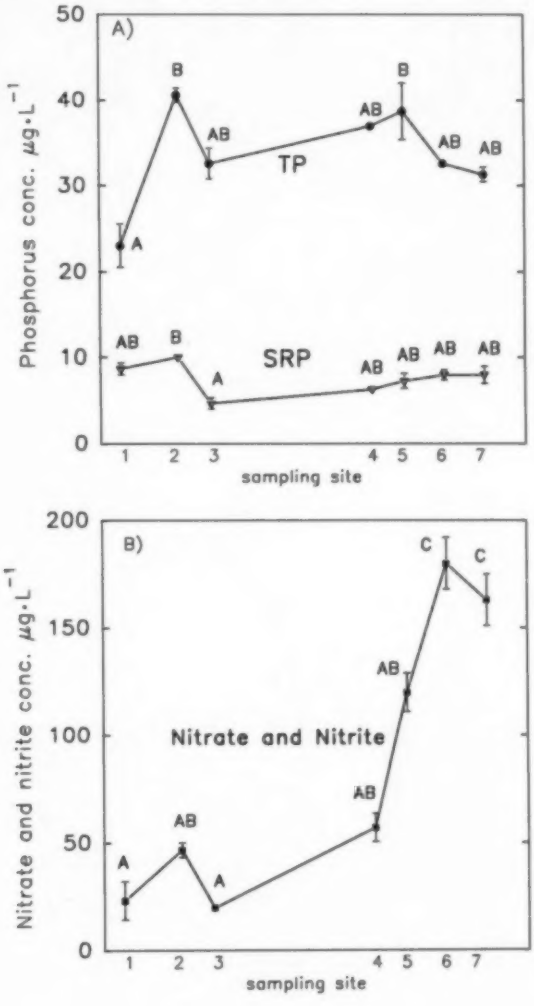
The mean TP concentration over the entire sampling period for all seven sites combined was 34.8 ± 0.88 $\mu g \cdot L^{-1}$ ($n = 125$). Non-parametric two-way ANOVA followed by Tukey-type multiple comparisons, indicated that sites in the Rideau River proper (sites 2–7) had significantly higher TP concentrations than the Rideau headwater site (site 1) (overall ANOVA, $F = 34.3$, $p < 0.0001$; for site, $F = 99.28$, $p < 0.0001$; for month, $F = 67.62$, $p < 0.0001$). There were generally no significant differences in TP concentration among any of the river sites (Figs. 5A and 6A for May and July, all other months exhibited a similar pattern). Seasonally, there was a significant increase in TP concentration from June to July and a significant decrease in TP concentration from August to September within the river sites (Fig. 7A for site 6, all other river sites exhibited a similar pattern). There were no significant differences

in TP concentration in Lower Rideau Lake (site 1) over the sampling period with a concentration of 22.2 ± 3.2 $\mu g \cdot L^{-1}$ (n = 18).

The overall SRP concentration in the sampled portion of the Rideau River was 8.2 ± 0.58 $\mu g \cdot L^{-1}$ (n = 126). As with TP, there were significant differences in SRP concentration among sites and among months (overall nonparametric two-way ANOVA, F = 84.69, $p < 0.0001$; for site, F = 255.61, $p < 0.0001$; for month, F = 116.69, $p < 0.0001$). No obvious spatial trends were apparent and seasonally SRP tended to vary as TP varied (Figs. 5A, 6A, and 7A).

The mean $NO_2 + NO_3$ concentration of all sample sites and months combined was 42.5 ± 4.3 $\mu g \cdot L^{-1}$ (n = 123). Nonparametric two-way ANOVA indicated significant differences in $NO_2 + NO_3$ concentration among sites and among months (overall ANOVA, F = 90.46, $p < 0.0001$; for site: F = 419.6, $p < 0.0001$; for month: F = 131.8, $p < 0.0001$). $NO_2 + NO_3$ concentrations were significantly higher at the downstream sites 6 and 7 than in the remainder of the river (Figs. 5B and 6B) Seasonally, $NO_2 + NO_3$ concentrations were higher in May than in the other months. There were no significant differences from June to October (Fig. 7B).

Fig. 5. Longitudinal variation in (A) total phosphorus (TP) and soluble reactive phosphorus (SRP) concentrations and (B) nitrate and nitrite concentration in the Rideau River during May 1993. Plotted are means ± SE, n = 3. Means with same letter are not significantly different at 5%. Sites are those identified in Fig. 1.



Determinants of algal biomass

Multiple regression analysis was used to identify which (if any) combination of the independent variables could best be used to predict chl-*a* concentration within the Rideau River. The dependent variable chl-*a* concentration and the independent variables TP concentration, SRP concentration, and $NO_2 + NO_3$ concentration were log transformed to achieve normality (Wilkes-Shapiro, p = 0.201, n = 107) and homoscedasticity (p = 0.259, n = 107) prior to multiple regression analysis. For the multiple regression, log chl-*a* concentration was the dependent variable and log TP concentration, log $NO_2 + NO_3$ concentration, log SRP concentration, temperature, and discharge were the independent variables. A correlation matrix (Pearson product moment correlation) revealed that, among the independent variables, log TP concentration was significantly and highly positively correlated with temperature, the correlation coefficient being 0.615 (Table 1). Discharge was significantly negatively correlated with temperature and positively with log $NO_2 + NO_3$ concentration. Log SRP concentration was significantly positively correlated with log TP concentration, temperature, and log $NO_2 + NO_3$ concentration. These later correlations, though significant, were not high (correlation coefficients all below 0.60; Table 1). The most suitable multiple regression equation (i.e., with the highest coefficient of determination, all regressors significant, and limited multicollinearity) was log[chl-*a*] = −0.95 + 1.41 log[TP] + 0.094 log[$NO_2 + NO_3$] − 0.560 log[SRP] ($p < 0.001$, R^2 = 0.51, RMS = 0.029).

This multiple regression equation, though significant, is difficult to interpret. The positive relationship of chl-*a* concentration with TP and $NO_2 + NO_3$ concentration is understandable, but, the negative relationship with SRP concentration is not. Neither discharge nor temperature were significant regressors. Of the variance in log chl-*a* concentration accounted for by this multiple regression, 71% was contributed by the log TP concentration term. Therefore, to obtain a simpler and more interpretable equation, a simple linear regression was performed between the logarithms of the chl-*a* and TP concentrations.

Average chl-*a* and TP concentrations for river sites were log transformed to achieve normality (Wilkes–Shapiro, p = 0.18, n = 36) and homoscedasticity (p = 0.186, n = 36) prior to linear regression of chl-*a* with TP. Results of the linear regression analysis indicated that log chl-*a* concentration tended to increase as log TP concentration increased (p = 0.016, R^2 = 0.158, RMS = 0.049) (Fig. 8). However,

Fig. 6. Longitudinal variation in (A) total phosphorus (TP) and soluble reactive phosphorus (SRP) concentrations and (B) nitrate and nitrite concentration in the Rideau River during July 1993. Plotted are means ± SE, $n = 3$. Means with same letter are not significantly different at 5%. Sites are those identified in Fig. 1.

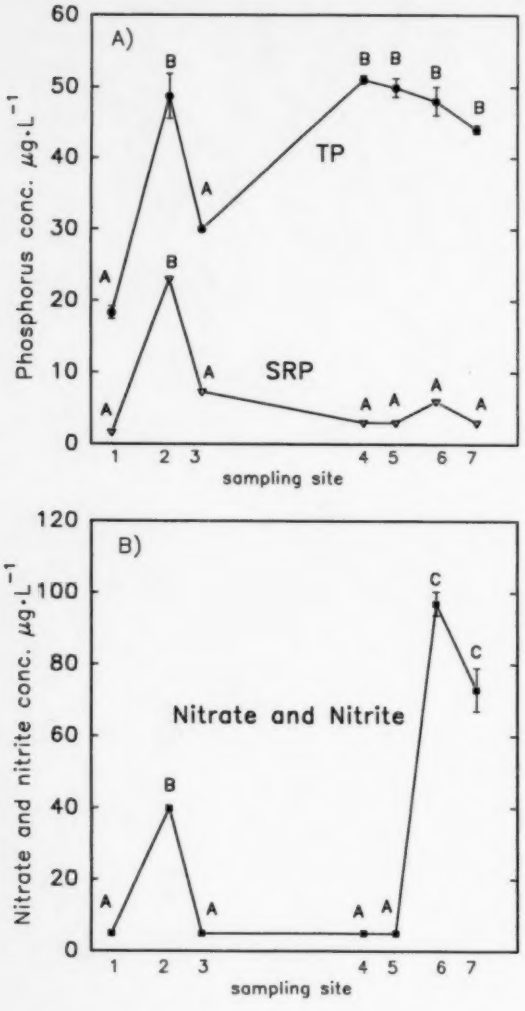
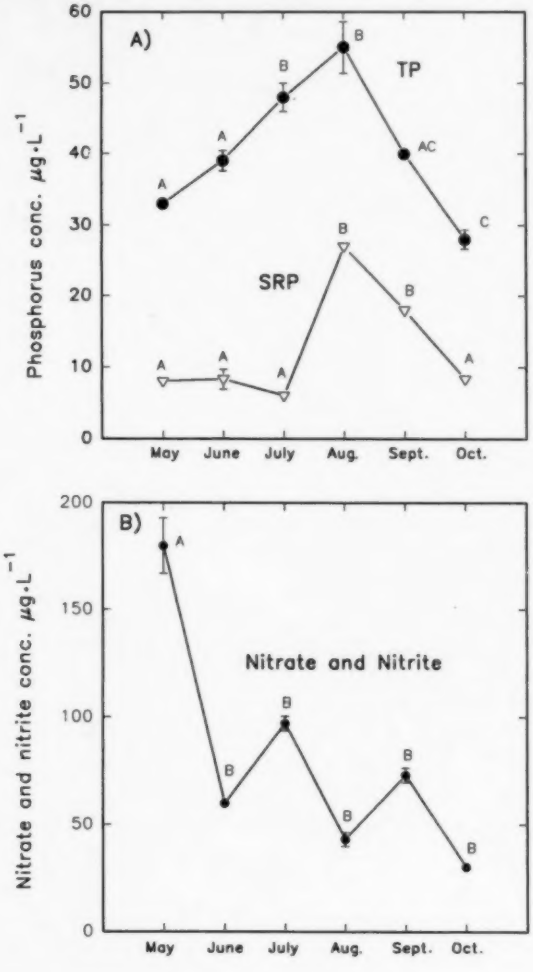


Fig. 7. Seasonal changes in (A) total phosphorus (TP) and soluble reactive phosphorus (SRP) concentrations and (B) nitrate and nitrite concentrations for site 6 on the Rideau River in 1993. Plotted are means ± SE, $n = 3$. Means with same letter are not significantly different at 5%.



this regression was significant only with the inclusion of a single point with low TP concentration and low chl-*a* concentration (indicated in Fig. 8). Removal of this point resulted in no detectable relationship between log chl-*a* concentration and log TP concentration ($p = 0.162$, $R^2 = 0.058$, RMS = 0.046).

Algal biomass change through the defined reaches

Retention times of the river reaches designated as long, medium, and short retention varied as discharge varied seasonally (Table 2). Through the reach with a long retention time (between site 2 and site 3) chl-*a* concentration increased in May, August, September, and October and remained unchanged in June and July (Fig. 2). Through the reach with a medium retention time (between site 6 and site 7) chl-*a* concentration increased significantly in all months except May and October (Fig. 2). Through the reach with a short retention time (between site 4 and site 5) there was never an increase in chl-*a* concentration and significant decreases in concentration occurred in May and October (Fig. 2).

Rates of biomass change calculated from the change in chl-*a* concentration from inflow site to outflow site of each river reach (Kohler 1993) indicated that net increases only occurred through the reaches with long and medium retention times (Table 3). In the reach with short retention time no increases occurred and net loss occurred

Table 1. Correlations between log TP concentration, discharge, temperature, log SRP concentration, and log NO_2 + NO_3 concentration for the Rideau River. Pearson product moment correlation coefficients with probabilities in parentheses (n = 107).

	Discharge	log [SRP]	log [NO_2 + NO_3]	Temperature
log [TP]	−0.038	0.414	0.109	0.615
	(0.699)	(<0.001)	(0.262)	(<0.001)
Discharge		−0.044	0.452	0.257
		(0.653)	(<0.001)	(0.008)
log [SRP]			0.415	0.369
			(<0.001)	(<0.001)
log [nitrate + nitrite]				−0.105
				(0.284)

during May and October (Table 3). In the reach with medium retention rate of biomass change was low in May, increased to a maximum in August and decreased in September and October (Table 3).

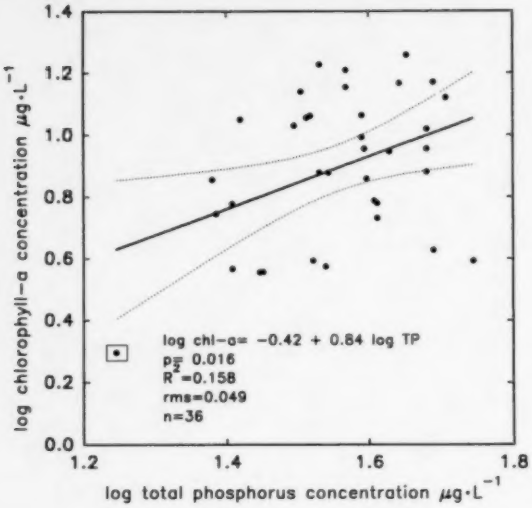
Discussion

Considerable longitudinal variation in chl-*a* concentration was observed in the Rideau River. Results clearly showed large increases and decreases in chl-*a* concentration between river sites. Longitudinal heterogeneity appears to be a characteristic feature of chl-*a* concentrations within riverine environments (Capblancq and Descamps 1978; Baker and Baker 1979; Moss et al. 1984; Soballe and Bachman 1984; Soballe and Threlkeld 1985; Chessman 1985; Dauta 1986; de Ruyter van Steveninck et al. 1990*a*; Pace et al. 1992; Kohler 1993). In the Rideau River, chl-*a* concentrations did not simply increase gradually downstream as has been observed in other rivers (Greenberg 1964; Capblancq and Descamps 1978; Holmes and Whitton 1981). In the Rideau there were distinct stretches of river over which net increase and net decrease occurred. Dauta (1986) observed that in the River Lot, overall longitudinal development of phytoplankton was marked by phases of increase and phases of decline. Jones (1984), Kohler (1993), and Cole et al. (1992) also observed localized river reaches over which algae increased or declined. In the Hudson River, Pace et al. (1992) isolated reaches of net population growth and net population loss when examining zooplankton communities. From the results of the present study and those cited above, it appears that planktonic populations do not simply increase gradually with downstream flow. As Reynolds (1988) suggests, downstream changes in algal biomass may occur in a stepwise manner in which there are reaches of net growth, reaches of net loss, and reaches of no change. The ability to detect such reaches may depend on the scale at which observations are taken. In studies in which reach length has been a few kilometres or tens of kilometres, localized increases or decreases in planktonic populations have been observed (Jones 1984; Dauta 1986; Pace et al. 1992; Cole et al. 1992; Kohler 1993; and the present study). Other studies covering hundreds or thousands of river kilometres tend to indicate more gradual changes in planktonic populations (e.g., Greenberg 1964; Talling and Rzoska 1967).

The initial decrease in chl-*a* concentration on movement from site 1 to site 2 has been similarly observed in other lake–river transitions. Talling and Rzoska (1967) suggest interception of plankton by submerged vegetation and loss due to benthic filtration as possible mechanisms to account for the decreases in plankton concentration on movement from a lake into a river. The outflow of Lower Rideau Lake has an extensive development of aquatic macrophytes, which may contribute to the decrease in chl-*a* concentration by intercepting outflowing phytoplankton. Kohler (1993) suggests that algae that develop in lentic water are ill-suited to lotic conditions. Uehlinger (1993) concluded that physiological damage occurred to fragile lake phytoplankton on movement into the more turbulent River Glatt. In the Rideau the initial decrease in chl-*a* concentration after 14 km of river travel resulted in low chl-*a* concentrations at site 2 for all months. This low chl-*a* concentration occurred despite high nutrient concentrations.

From site 2 to site 3 (the reach with long retention), a significant increase in chl-*a* concentration occurred in 4 of the 6 months sampled. The retention time of this reach was a minimum of 72 h during the high discharge of May and during other months it was from 1–2 weeks. As the retention time of a body of water increases, the effects of advection on the planktonic community decrease (Soballe and Kimmel 1987; Pace et al. 1992). Soballe and Threlkeld (1985) observed significant increases in chl-*a* concentration between the inflow and the outflow of a river impoundment with a mean retention time of 2.4 d. Retention times within the long retention reach of the Rideau were always sufficiently long for increases in chl-*a* concentration to occur. In addition, the average depth of this reach was approximately 2 m. This shallow depth likely meant that as algal cells circulated through the water column, they received sufficient light for positive growth. Attenuation coefficients in the Rideau are typically less than 1.5 m^{-1} (B.K. Basu, unpublished data). This would allow for a euphotic depth (1% light level) of approximately 3 m. Cole et al. (1992) hypothesized that algal blooms in the Hudson River could only occur where the depth was less than 4 m. At greater depths, phytoplankton was carried below the photic zone (by turbulent mixing) and remained below the 1% light level for a period of time long enough to make net positive algal growth impossible (Cole et al. 1992). In the Rideau, this type of light limitation is not

Fig. 8. Relationship between log [TP] and log [chl-*a*] for monthly means of Rideau River sites. Dotted lines are the 95% CIs. Relationship is significant only with the inclusion of boxed point (see text).



likely. Shallow depths result in the majority of the water column (sometimes the entire water column) being in the euphotic zone. Sufficient retention time and sufficient light availability (due to a shallow depth) resulted in net increases in chl-*a* generally occurring through the reach with long retention time. This initial development of algae may act to inoculate the river with potamoplankton. Kohler (1993) observed that the main flow of the River Spree was inoculated with algae originating from slower flowing reaches. The lack of increase in chl-*a* concentration in June and July from site 2 to site 3 parallels the clear water phase that occurred in Lower Rideau Lake (site 1) at this time. Clear water phases have been observed for other rivers following a spring bloom (Chessman 1985; Bothar and Kiss 1990; Cole et al. 1992; Kohler 1993).

In all months there was a significant increase in chl-*a* concentration from site 3 to site 4 (approximately 40 km). There was clearly positive net growth over this section of the river. Chessman (1985) observed similar results in the La Trobe River, in which an inoculum of algae originating from a midriver impoundment continued to increase downstream. From the decrease in chl-*a* concentration observed from site 1 to site 2 and the subsequent increases from site 2 to site 3 and site 3 to site 4, we conclude that a true potamoplankton developed within the Rideau River. The algae in the river were not simply a reflection of the algae in the headwaters. Similarly, Lewis (1988) concluded that most of the phytoplankton biomass in the Orinoco river originated from within channel sources, not from floodplain lakes. Once a significant chl-*a* concentration was established in the Rideau by site 4 (km 62) there was never an increase from site 4 to site 5 (the reach with a short retention time). The retention time of this reach was at most 58 h and was often under 40 h. Therefore, simple time limitation may have limited any increase in chl-*a* concentration over this reach with a short retention time. Increases in chl-*a* concentration over this reach were never observed as retention time was never sufficiently long. The average depth of this reach was approximately 3 m. Once again, this shallow depth meant that phytoplankton were likely not circulated below the photic zone and not light limited.

The decrease in chl-*a* concentration that occurred from site 5 to site 6 remains the most puzzling longitudinal trend in the Rideau River. There are no obvious sedimentation areas within this section of river. The river deepens from approximately 3 m average depth to 5 m average depth between these sites. However, the possibility of light limitation again seems unlikely with a euphotic depth of 3 m. In a turbid river (the Hudson), with attenuation coefficients from 2 to 4 m^{-1} Cole et al. (1992) determined that algal respiration would be greater than photosynthesis (i.e., net loss) when the depth became greater than approximately 7 m. In the Rideau, the depth at which photosynthesis equalled respiration would likely be greater than 7 m because of less nonbiogenic turbidity.

Another cause of the decrease in chl-*a* concentration from site 5 to site 6 may have been herbivore grazing on algae, as de Ruyter van Steveninck et al. (1992) observed in the River Rhine. This would be possible had a large zooplankton community developed over this stretch of the Rideau. Lacking data concerning zooplankton biomass, however, we could not determine the effect of zooplankton grazing on algal biomass.

From site 6 to site 7 (the reach with medium retention) significant increases in chl-*a* concentration were consistently observed. It appeared that the retention time through the reach with medium retention was always sufficient (except in May) for an increase in algal biomass to be observed. The average depth of this reach was approximately 5 m, so light limitation is again unlikely.

Though chl-*a* concentration in the Rideau River was significantly related to TP concentration, the low coefficient of determination for this regression ($R^2 = 0.158$) indicated that much variability remained unexplained and removal of a single point eliminated any significant relationship between chl-*a* concentration and TP concentration. It was clear that when nutrient concentrations (TP, SRP, and $NO_2 + NO_3$) did not vary significantly from site to site, chl-*a* concentration did vary from site to site. Soballe and Kimmel (1987) studying the large NES and NASQUAN data sets concluded that algal abundance in rivers was significantly less predictable than in natural lakes. In our study, R^2 was much lower for the river data ($R^2 = 0.158$) than for published chl-*a* – TP regressions based on lake data (e.g., Dillon and Rigler 1974). Soballe and Kimmel (1987) showed algal abundance per unit phosphorus increasing in the sequence: rivers < impoundments < natural lakes. When we included the SRP and $NO_2 + NO_3$ terms in the equation predicting chl-*a* concentration the coefficient of determination was still relatively low ($R^2 = 0.51$). Other factors in addition to nutrient concentrations may determine chl-*a* concentration in the Rideau River.

We observed no significant relationship between chl-*a* concentration and discharge in the Rideau River. This runs counter to most other river studies in which algal biomass

Table 2. Calculated theoretical retention times of Rideau River reaches. Retention times calculated as reach volume divided by river discharge for period 7 d prior to and including sampling date.

River reach	Retention time (h)					
	May	June	July	Aug.	Sept.	Oct.
Long retention (site 2 to site 3)	72	198	218	395	367	271
Medium retention (site 6 to site 7)	19	67	74	154	120	103
Short retention (site 4 to site 5)	8	26	29	58	45	39

Table 3. Changes in chl-*a* concentration (Δ chl-*a*; $\mu g \cdot L^{-1}$) from inflow site to outflow site and calculated rates of change of biomass in ln units per day (k) for Rideau River reaches of varying retention time.

Month	River reach					
	Long retention (site 2 to site 3)		Medium retention (site 6 to site 7)		Short retention (site 4 to site 5)	
	Δ chl-*a*	k	Δ chl-*a*	k	Δ chl-*a*	k
May	6.03	0.25	ns	0	−3.21	−0.66
June	ns[a]	0	2.41	0.07	ns	0
July	ns	0	4.26	0.10	ns	0
August	3.77	0.04	5.26	0.14	ns	0
September	4.03	0.05	3.68	0.13	ns	0
October	3.38	0.09	ns	0	−5.53	−0.45

[a]ns, nonsignificant change in chl-*a* concentration and a net growth rate of zero.

is negatively correlated with discharge (summarized by Reynolds 1988). The absence of a relationship between chl-*a* and discharge may be due to the limited range of discharges over which sampling was conducted. In the Rideau, the maximum discharge occurs in April and high discharge occurs in November. By sampling from May to October we failed to sample during these high discharge periods. Bothar and Kiss (1990), working on the Danube concluded that, from May until October, water discharge conditions were always favourable for potamoplankton development. They also may have sampled over too limited a range of discharges to observe any discharge effects.

In this study, the rates of biomass increase through the reaches with long and medium retention time were low (usually under 0.15 d^{-1}. Positive rates of biomass change (increases) only occurred through the long and medium retention reaches. In the reach with short retention time, no increases were observed. It would appear that within the reaches with long and medium retention, sufficient time was available for potamoplankton to increase in concentration. The shallow depth of these reaches (2 m and 5 m) likely prevented circulation of algal cells below the photic zone and associated light limitation. The reach with a short retention time, though also shallow (3 m), did not act as a potamoplankton source. The water in this reach may have been retained for too short a time for any increase in chl-*a* concentration to occur.

To summarize, chl-*a* concentrations within the Rideau River displayed considerable longitudinal variation. There appeared to be reaches of net increase in algae (sources), reaches of no change, and reaches of net decrease (sinks). Chlorophyll *a* did not simply increase gradually downstream and may develop in a stepwise manner within the Rideau. We were able to identify reaches of net algal growth and net algal loss. Chlorophyll *a* concentration only increased over the reaches with long and medium retentions, never over the reach with short retention times. The shallow depths of all three reaches makes it unlikely that algal cells were circulated below the photic zone for a period of time sufficient to limit growth. At best, there exists a weak positive relationship between chl-*a* concentration and TP concentration in the Rideau River. A limited range of discharges may be responsible for the lack of a significant relationship between chl-*a* and discharge. This suggests that additional abiotic or biotic factors may also regulate algal biomass in the Rideau River. Continued research including the parameters of light availability, zooplankton biomass, and grazing is required.

Acknowledgements

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Episodic flow–duration analysis: a method of assessing toxic exposure of brook trout (*Salvelinus fontinalis*) to episodic increases in aluminum

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Abstract: Episodic flow–duration curves were developed that summarize stream flow data by considering the duration of sustained flows at or above specified levels. These curves and relationships between dissolved aluminum concentrations and stream flow were combined for five Appalachian (Pennsylvania) streams to determine the frequency of occurrence of acutely toxic aluminum conditions for brook trout (*Salvelinus fontinalis*). Concentrations of total dissolved aluminum (Al_{TD}) increased with stream flow in all streams, but reached a plateau or decreased during extreme high flows in some streams. Al_{TD} was dominated by inorganic monomeric species in all streams except Benner Run and Baldwin Creek. Episodic Al_{TD} concentrations and durations were compared with a toxicity curve developed using caged wild and hatchery-reared brook trout. Results predicted that lethal episodes occurred on Linn, Stone, and Roberts runs, but not on Benner Run and Baldwin Creek. With the exception of Stone Run, brook trout populations were largest in streams without predicted lethal episodes (Benner Run and Baldwin Creek) and were smallest in streams with predicted lethal episodes (Linn and Roberts runs). This analysis could be adapted for prediction of toxic conditions in streams for other pollutants where toxicity varies with both concentration and duration of exposure.

Résumé : On a produit des courbes épisodiques des débits classés résumant les données sur les débits de cours d'eau en tenant compte de la durée de débits soutenus à des niveaux donnés ou au-dessus. Ces courbes et les liens entre les concentrations d'aluminium dissous et le débit des cours d'eau des cours d'eau ont été combinés pour cinq cours d'eau des Appalaches (en Pennsylvanie), dans le but d'établir la fréquence d'apparition de concentrations d'aluminium toxiques à effets aigus pour l'omble de fontaine (*Salvelinus fontinalis*). Les concentrations d'aluminium total dissous (Al_{TD}) ont augmenté avec le débit dans tous les cours d'eau et ont atteint un plateau ou ont décru lors de débits élevés extrêmes dans certains cours d'eau. L'Al_{TD} était dominé par la forme inorganique monomère dans tous les cours d'eau à l'exception de Benner Run et Baldwin Creek. Les concentrations épisodiques d'Al_{TD} et leurs durées ont été comparées à une courbe de toxicité élaborée à l'aide d'ombles de fontaine sauvages et d'élevage gardées en cages. Les résultats laissent prévoir que des épisodes létaux se produiront dans les ruisseaux Linn, Stone, Roberts, mais pas dans Benner Run ni Baldwin Creek. À l'exception de Stone Run, les populations d'omble de fontaine étaient maximales dans les cours d'eau où aucun épisode létal n'est à prévoir (Benner Run et Baldwin Creek) et minimales dans les cours d'eau où des épisodes létaux sont à prévoir (Linn et Roberts). Cette analyse pourrait être adaptée pour la prévision de conditions toxiques présentées dans des cours d'eau par d'autres polluants, où la toxicité varie en fonction de la concentration et de la durée de l'exposition.
[Traduit par la Rédaction]

Introduction

The occurrence of episodes of acid runoff has been well documented in numerous studies of streams in North America and Europe (Wigington et al. 1990, 1992, 1993). In the northeastern United States these episodes are normally accompanied by declines in acid neutralizing capacity (ANC) and pH and measurable increases in total dissolved aluminum (Al_{TD}) concentrations (Schaeffer et al. 1990; Gagen et al. 1993; Herlihy et al. 1993; O'Brien et al. 1993;

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DeWalle and Swistock 1994). Numerous studies on headwater streams of the northern Appalachian Plateau region of Pennsylvania have reported strong positive relationships between stream flow and Al_{TD} concentrations (Sharpe et al. 1984; Swistock et al. 1989; Carline et al. 1992; Gagen et al. 1993). Increased Al_{TD} concentrations during episodes of acid runoff are especially important because many fish species native to this region are sensitive to elevated aluminum concentrations (Baker and Schofield 1982; Sharpe et al. 1983; Baker et al. 1990; Fiss and Carline 1993; Gagen et al. 1993).

Dissolved aluminum in streams on the northern Appalachian Plateau is generally believed to originate from the soil. Forest soils in this region are derived mostly from acidic sandstones or shales and soil water contains relatively high levels of Al_{TD} (DeWalle et al. 1988). The region also experiences some of the most acidic wet deposition in the United States (Knapp et al. 1988), which probably has contributed to soil acidity and high Al_{TD} concentrations in soil water. During rain and snowmelt events, soil water is flushed into the stream channel causing stream concentrations of Al_{TD} to generally increase with stream flow (Swistock et al. 1989; DeWalle and Balliet 1992). Hydrologic studies with chemical and isotopic tracers have shown that soil water can contribute from 16 to 47% of total flow during runoff events (DeWalle and Pionke 1994). During large runoff episodes, precipitation or meltwater that moves rapidly over the watershed surface to the channel without contacting the soil contains negligible Al_{TD} and may serve to dilute stream concentrations (Dow et al. 1994). Deep groundwater, which sustains stream flow during baseflow and contributes to flow during acidic episodes, generally contains low Al_{TD} concentrations (Sharpe et al. 1984). Stream flow Al_{TD} during acidic episodes is the result of a dynamic mixture of varying fractions of precipitation, soil water, and deep groundwaters (DeWalle et al. 1988; Swistock et al. 1989). Watershed physiographical, biological, and geochemical characteristics control the fractions and Al_{TD} concentrations of source waters that mix to create stream flow. Currently, processes are not well enough understood to model or predict stream flow Al_{TD} concentrations in streams, and reliance is placed on direct measurement.

Previous research has shown that aluminum toxicity to fish is a complex process. Concentrations of fluoride and dissolved organic carbon (DOC) in a stream can each affect the toxicity of aluminum to fish by forming aluminum complexes that are less toxic (Driscoll et al. 1980; Miller and Andelman 1986; Baker et al. 1990; Simonin et al. 1993). In streams where DOC and fluoride concentrations are low, most of the Al_{TD} is present as the inorganic monomeric species (Miller and Andelman 1986), which has been shown to be responsible for stress and mortality of fishes (Driscoll et al. 1980; Clark and LaZerte 1985). Numerous studies have also established that calcium in water ameliorates aluminum toxicity presumably by influencing membrane permeability within the fish (McDonald 1983; Booth et al. 1988).

In addition to absolute concentrations of Al_{TD}, fluoride, DOC, and calcium, the duration of aluminum exposure has been identified as an important factor affecting fish mortality during acidic episodes (Gagen and Sharpe 1987; Klauda and Palmer 1987; Hutchinson et al. 1989; Schweinforth et al. 1989; Simonin et al. 1993). Most studies on the length of exposure to aluminum have involved laboratory bioassays rather than field experiments, although one field experiment in Pennsylvania showed that at least 24 h of exposure to Al_{TD} concentrations in excess of 200 $\mu g \cdot L^{-1}$ were necessary to cause mortality of caged, hatchery-reared, yearling brook trout (Gagen and Sharpe 1987).

The objective of many of the aforementioned studies has been to either predict or explain fish mortality that might occur based on observed mortality in bioassays and either measured or predicted concentrations of important chemical parameters. Because it is difficult to sample streams continuously for Al_{TD} and other important chemical parameters, it is necessary to model episodic toxicity by predicting chemical parameters from stream flow data and concentration–flow relationships. There is an additional need, however, to incorporate the effects of duration of exposure into such models by characterizing the flow regimes of streams. With such a method, long-term measurements of stream flow could be used to predict exposures of target organisms to toxic substances.

In this paper we develop episodic flow–duration curves to account for both the frequency and duration of sustained stream flows. Using episodic flow–duration curves, streams with long-duration flow events can be differentiated from streams with short-duration or "flashy" flow events. The objective of this paper is to use episodic flow–duration curves in conjunction with the relationship between Al_{TD} concentration and stream discharge to determine toxicity to brook trout in five streams on the northern Appalachian Plateau. Results are then compared with observed brook trout populations in each stream.

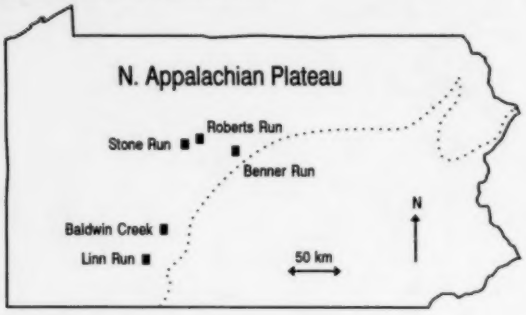
Materials and methods

Study areas

The five study watersheds are located in the northern portion of the Appalachian Plateau Province of Pennsylvania in the northeastern United States (Fig. 1). These streams, along with eight others in the Catskill and Adirondack regions of New York, were part of the U.S. Environmental Protection Agency (USEPA) sponsored Episodic Response Project (ERP). The ERP project was designed to intensively monitor stream hydrochemical and fisheries responses during acidic episodes. The five Pennsylvania watersheds were selected to represent a gradient in the severity of acid episodes based on preliminary site surveys. Benner Run and Baldwin Creek were chosen as reference watersheds because they have higher baseflow ANC and experience less severe acidic episodes with lower maximum Al_{TD} concentrations. Stone, Roberts, and Linn runs all have lower baseflow ANC and experience more severe acidic episodes with higher Al_{TD} concentrations.

The five basins are representative of forested, unglaciated watersheds in this region with low conductivity, second-order streams draining basins of 535–1156 ha. Bedrock in all five basins is sandstone and shale. The region has a humid continental climate and receives approximately 100 cm of annual precipitation. Snowpacks are intermittent

Fig. 1. Location of the study watersheds within the northern Appalachian Plateau region of Pennsylvania in the northeastern United States.



on the study watersheds, and no significant accumulations occurred during the study period. Electrofishing surveys indicated that brook trout (*Salvelinus fontinalis*) inhabit the study sections of all five streams. Mottled sculpins (*Cottus bairdi*) are also present in Baldwin Creek while slimy sculpins (*Cottus cognatus*) and brown trout (*Salmo trutta*) occur in Benner Run. More detailed information on the geology, soils, physical characteristics, and aquatic biota of the study watersheds is available elsewhere (DeWalle et al. 1993; Gagen et al. 1993).

Episodic flow–duration curves

Stream stage or depth data were collected every hour from October 1988 through April 1990 on all streams using FW-1 water level recorders with potentiometric output to Campbell CR-10 data loggers. Rating curves relating logarithm of stream flow to logarithm of stream stage (R^2 = 0.90–0.99) were developed by gaging the streams with current meters to convert continuous stage records to flow for each stream. Stream flow rates in cubic meters per second were normalized by dividing by basin area in square kilometers to give flows in cubic meters per second per square kilometer to facilitate comparisons of data among basins of different areas.

The episodic flow–duration curves for each stream were developed by using a frequency distribution procedure in the SAS statistical package (SAS Institute Inc. 1985). Initially, a frequency distribution was obtained of the sustained durations of flows in each stream that were at or above specified flow rates during the period of record. Flow rates were chosen to represent the distribution of high flows occurring in a stream during acidic episodes when toxic conditions were likely to occur. The maximum duration at or above each specified flow rate was then plotted on a graph of stream flow rate versus duration of flow. A line connecting the points of maximum duration for each flow rate was used to define the episodic flow–duration curve for that stream (Fig. 2).

Durations up to only 400 h (16.7 d) were considered in developing the curves because greater durations were not considered to be biologically relevant for exposure of brook trout to Al_{TD}. Additionally, caged fish experiments, on which mortality estimates were based, only lasted for 20 d. Maximum durations for very low flow rates, which were not considered here, would be very large. For example, the lowest observed stream flow during the study would have a duration equal to the period of record (566 d).

The solid line on each episodic flow–duration curve represents the maximum duration (one occurrence) for which each stream flow occurred during the period of record (Fig. 2). Such episodic flow–duration curves are considered to be the most biologically relevant, because maximum flow–duration conditions that occur only once in the stream during the life of an organism can be lethal. However, if repeated occurrences of certain combinations of high flow and duration were also found to be biologically important, then curves describing flow–duration combinations which occurred with greater frequency, such as 3, 5, or 10 times during the period of record, could be constructed.

Relationships between aluminum and stream flow

Samples of stream water were collected intensively during acidic episodes using automatic water samplers, while baseflow samples were collected by hand. Water samples were analyzed at the Water Laboratory, Environmental Resources Research Institute, Pennsylvania State University, University Park. Al_{TD} was determined, after filtering samples through 0.1-μm Millipore filters, by atomic absorption spectrophotometry according to USEPA (1983) recommended procedures. Approximately 25% of the samples were filtered using 0.22-μm Gelman cellulose acetate filters and speciated for aluminum based upon the methods of Barnes (1975) and Driscoll (1984), described in detail by Pagano (1990).

Rigorous quality assurance and quality control procedures were used to monitor data quality including the use of five monthly blind audit samples covering the range of field data (Peck et al. 1988). Mean precision (expressed as two times the standard deviations of low-level check samples) and weighted-mean bias (expressed as the absolute difference between measured and expected values for audit samples) for Al_{TD} were 0.037 and 0.045 $mg \cdot L^{-1}$ respectively.

Regression equations describing the relationship between Al_{TD} ($mg \cdot L^{-1}$) and stream flow rates ($m^3 \cdot s^{-1} \cdot km^{-2}$) for each stream were calculated using a nonlinear procedure in the SAS statistical package (SAS Institute Inc. 1985). The resulting equations were used to predict hourly Al_{TD} concentrations based upon hourly flow data from each stream. Sample sizes used for these regressions ranged from 287 to 414 samples per stream.

The regression equations were used to translate the episodic flow–duration curves into Al_{TD}-duration curves by predicting Al_{TD} equivalent to specified flow rates on the vertical axis of the episodic flow–duration curves. The flow rate scale was thus transformed into an Al_{TD} scale and the entire graph was transformed into an Al_{TD}-duration curve for each stream. The Al_{TD}-duration curves could then be used to determine the maximum duration of exposure of wild brook trout in each stream to specific Al_{TD} concentrations.

Al_{TD} rather than dissolved inorganic monomeric Al concentrations (Al_{IM}) were used to assess toxicity of aluminum to brook trout in this study. Al_{IM} has been found to be primarily responsible for toxic effects of Al (Driscoll et al.

Fig. 2. Episodic flow–duration curves for each stream for the period October 1988 to April 1990. The area beneath the solid curve represents observed conditions where stream flow was continuously equalled or exceeded for some duration. The solid line indicates the maximum duration of sustained stream flow equal to or greater than each specified flow rate during the period of record. The dotted line indicates the duration of sustained stream flows which equalled or exceeded specified flows three times during the study period.

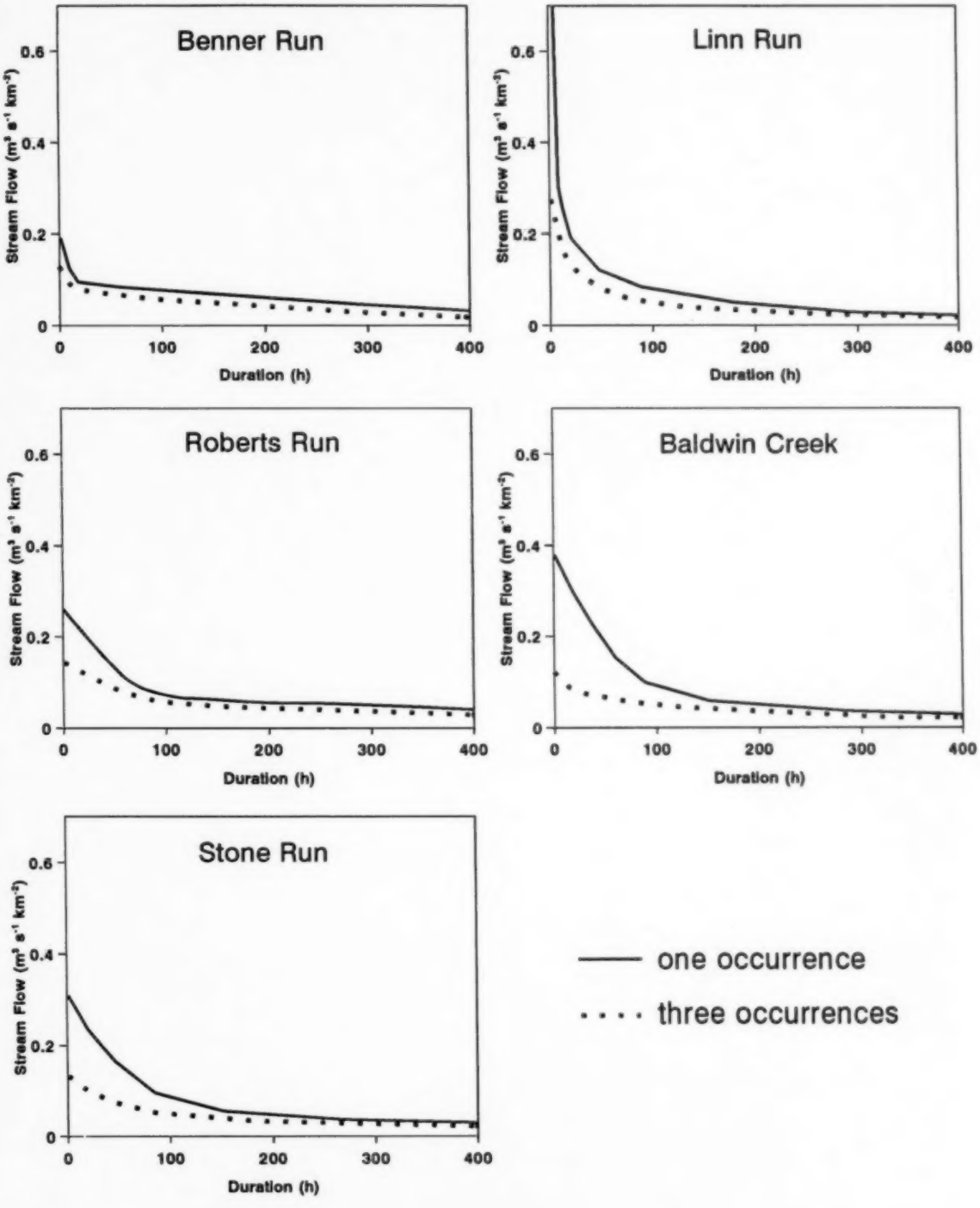


Fig. 3. Relationships between total dissolved aluminum and stream flow for each study stream. Solid lines indicate the regression lines (Eq. 1–5) fitted to the data. Note scale differences for stream flow rates among the streams.

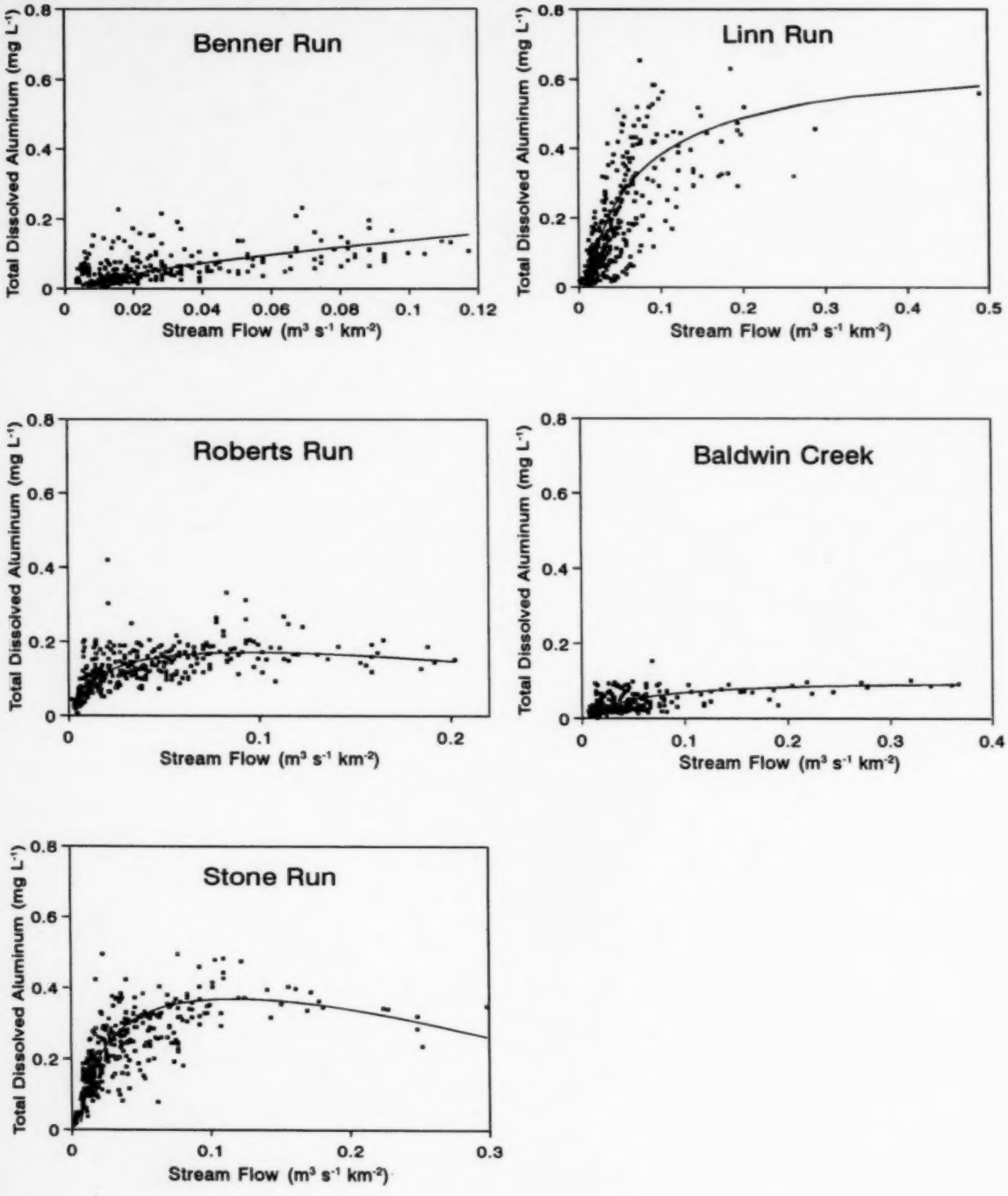


Table 1. Regression equations used to predict hourly Al_{TD} concentrations ($mg \cdot L^{-1}$) from stream discharge (Q, $m^3 \cdot s^{-1}\ km^{-2}$) and the $\log_{10}$ of stream flow ($\log Q$) for each stream.

Stream	N	Regression equation	R^2	p-value	Mean square error
Benner Run	333	$0.128 + 0.698(Q) + 0.060(\log Q)$	0.32	<0.0001	0.00126
Roberts Run	341	$0.405 - 0.727(Q) + 0.161(\log Q)$	0.56	<0.0001	0.00185
Stone Run	414	$0.924 - 1.502(Q) + 0.407(\log Q)$	0.84	<0.0001	0.00401
Linn Run	287	$0.859 - 0.283(Q) + 0.449(\log Q)$	0.75	<0.0001	0.00783
Baldwin Creek	318	$0.150 - 0.075(Q) + 0.074(\log Q)$	0.33	<0.0001	0.00038

1980; Baker et al. 1990). However, Al_{IM} and Al_{TD} concentrations were similar in the streams with high Al (Linn, Stone, Roberts runs) and a larger number of Al_{TD} than Al_{IM} samples were collected over a wider range of stream flows for all streams. Use of Al_{TD} caused little error in this study. In streams with high DOC concentrations and significant organic complexation, the use of Al_{IM} would have been necessary.

Fish populations

Fish populations were sampled by electrofishing in a 100-m-long section of each stream. Study sections were at least 600 m from any tributary that might have been a source of colonizing fish. Ten electrofishing surveys were conducted on each stream from March 1988 through June 1990: four surveys during spring (March–May), four during summer (June–August), and two during fall (November). Fish were captured, measured, and held during three to six electrofishing passes. Fish were redistributed after the last pass. The method developed by Zippen (1958) was used to calculate population numbers (±20%) for each survey at the 95% confidence level.

Acute aluminum toxicity to brook trout

Three to five experiments were conducted in each study stream to determine the sensitivity of brook trout to Al_{TD} during acidic episodes. Each experiment consisted of 17–35 trout, age 1 year or older, held in cages that were placed in the study streams for 20-d periods during spring and fall that encompassed numerous acidic episodes. The cages consisted of three to five 4-L polyethylene jugs with 2-mm-mesh fiberglass screening covering four 7 × 12 cm openings. Each jug contained 7 to 12 trout and there was always less than 35 g wet mass in each jug. The jugs were placed in wooden-framed cages covered with 12-mm-mesh Naltex screening. The cages were checked for fish mortality daily during acidic episodes and on alternate days during intervening low-flow periods. Dead fish were counted and removed from the cages. Detailed description of the caged fish experiments is available in Gagen et al. (1993).

Measurements of trout mortality during each acidic episode were combined with Al_{TD} concentration and duration data to determine which combinations of Al_{TD} and duration were lethal to brook trout. Episodes were defined, according to the method of Simonin et al. (1993), as continuous time periods with Al_{TD} concentrations above $100\ \mu g \cdot L^{-1}$. Al_{TD} values coincident with fish mortality were calculated as the median of hourly Al_{TD} concentrations from the initiation of the episode (when Al_{TD} first exceeded $100\ \mu g \cdot L^{-1}$) to the death of each fish or group of fish. Hourly Al_{TD} was predicted using the aforementioned regression equations relating Al_{TD} to stream flow. Exposure duration was calculated as the time (h) from initiation of the episode to the time of fish death and percent mortality was calculated as the percentage of total fish in cages that died during a specific episode. Calculations for a specific experiment were stopped when <10 fish remained in the wooden cage.

Information on nonlethal combinations of Al_{TD} and exposure duration were obtained from caged fish experiments in the reference streams (Benner Run and Baldwin Creek) where brook trout mortality was insignificant (Gagen et al. 1993). In the reference streams, episodes were defined as the time from the beginning of increased stream flow to the time when flow returned to the pre-episode level, because Al_{TD} concentrations rarely exceeded $100\ \mu g \cdot L^{-1}$. Al_{TD} concentrations, durations, and percent mortality were calculated as described above. Measurements of median Al_{TD} concentrations, exposure durations, and percentage mortality were combined with similar data reported by Gagen and Sharpe (1987) from caged fish experiments using hatchery-reared, yearling brook trout.

Results and discussion

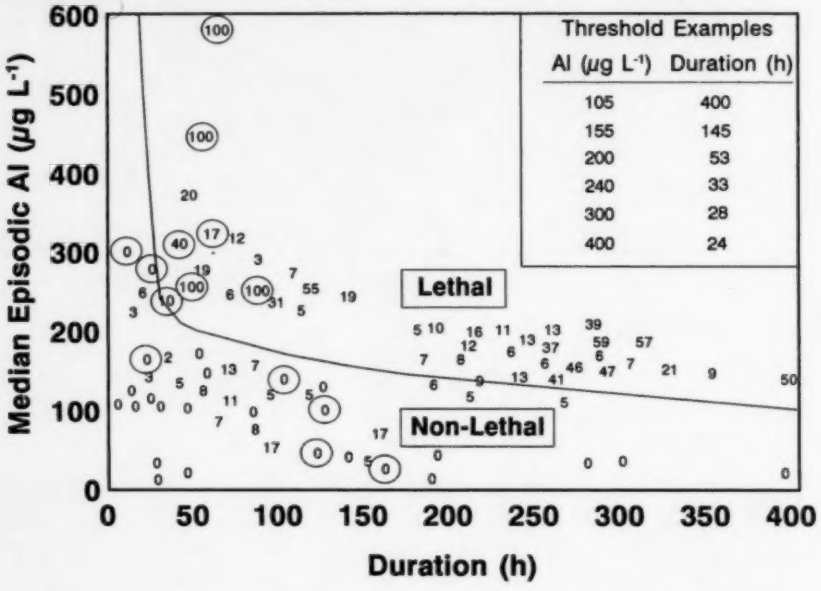
Episodic flow–duration curves

The region below the solid black line on each episodic flow–duration curve (Fig. 2) represents combinations of stream flow and duration that were observed during the study period. On Linn Run, for example, episodes with flows $\geq 0.2\ m^3 \cdot s^{-1} \cdot km^{-2}$ only occurred for durations of less than 10 h (Fig. 2). The solid line that defines the upper boundary of this region represents the maximum continuous duration that occurred for each stream flow during the 19-month study period.

The headwater nature of the five study streams resulted in generally flashy responses to rainfall events with numerous episodes occurring during the 19-month study period. Linn Run episodes were characterized by the highest flows and shortest durations of the five streams, whereas Baldwin Creek, Roberts Run, and Stone Run exhibited more moderate flows over longer durations (Fig. 2). Benner Run typically had the lowest flows during episodes.

The upper limit curve that defines the extreme combinations of stream flow and duration for each stream (solid

Fig. 4. Brook trout mortality in relation to median episodic Al_{TD} concentrations and durations of exposure. Numbers indicate the percentage mortality of wild brook trout in bioassays conducted during the ERP project (Gagen et al. 1993). Circled numbers are the percentage mortality from hatchery brook trout bioassays conducted by Gagen and Sharpe (1987). The solid curve is the approximate boundary line between lethal and nonlethal aluminum concentrations and durations given by Gagen and Sharpe (1987) extrapolated to 400 h duration.



line in Fig. 2) may be of greatest biological importance. Ideally, such curves would be derived from longer term records spanning the life expectancy of key organisms, rather than 19 months of hourly flow data as in this study. In some cases, repeated occurrences of episodes with high flow and duration may be of greater biological significance than the extreme case. Stream flow records can be used to generate curves for any number of occurrences of episodes with a specified flow and duration. The episodic flow–duration curves for the combination of flows and durations that occurred three times during the period of record are also given in Fig. 2 (dotted line) as an example. The three-occurrence curves were similar among the five streams except for Linn Run where higher flow rates occurred for durations less than about 25 h.

Aluminum–flow regression equations

Concentrations of Al_{TD} were closely related to stream flow but the magnitude and shape of the responses were varied among the five streams (Fig. 3). Regression equations were developed from these data to predict Al_{TD} in each stream from stream flow measurements (Table 1). Although several nonlinear functions could be fitted to the observed data, the stream flow (Q) and log stream flow (log Q) prediction variables provided the best fit to the data trends.

Al_{TD} concentrations generally followed a gradient with Linn Run > Stone Run > Roberts Run > Benner Run > Baldwin Creek. On all streams, except perhaps Benner Run, Al_{TD} concentrations reached a plateau or even decreased at higher stream flows (Fig. 3). Dilution behavior on Stone and Roberts runs indicates that toxic Al_{TD} conditions would occur most frequently on these streams at intermediate, rather than extreme high flows. Al_{TD} dilution could be related to relatively large volumes of low-Al_{TD} surface rain and meltwater added to these streams at high flows as previously discussed.

Al_{TD} concentration–duration toxicity curve for brook trout

Brook trout mortality data from the caged fish experiments are plotted with the concurrent episodic Al_{TD} concentrations and durations of exposure that preceded the mortality in Fig. 4. Additional data from previous caged fish experiments (circled numbers) and the toxicity curve separating the lethal and nonlethal zones are taken from Gagen and Sharpe (1987) and are shown for comparison. The original toxicity curve (Gagen and Sharpe 1987) was only reported to a duration of 200 h and was extrapolated to 400 h for this comparison.

The curve given by Gagen and Sharpe (1987) for hatchery-reared brook trout appears to also be a reasonable threshold for wild brook trout studied in the ERP. Nearly all of the periods with mortality greater than 10% fell within the lethal zone of the curve and most of the periods with mortality less than 5% were in the nonlethal zone. Overall, the data indicate that Al_{TD} in excess of 200 $\mu g \cdot L^{-1}$

Fig. 5. Episodic aluminum concentration-duration curves for each stream (dotted line) in comparison with the brook trout acute toxicity curve (solid line) from Gagen and Sharpe (1987). The hatched areas where these curves overlap represent the occurrence of potentially lethal conditions to brook trout.

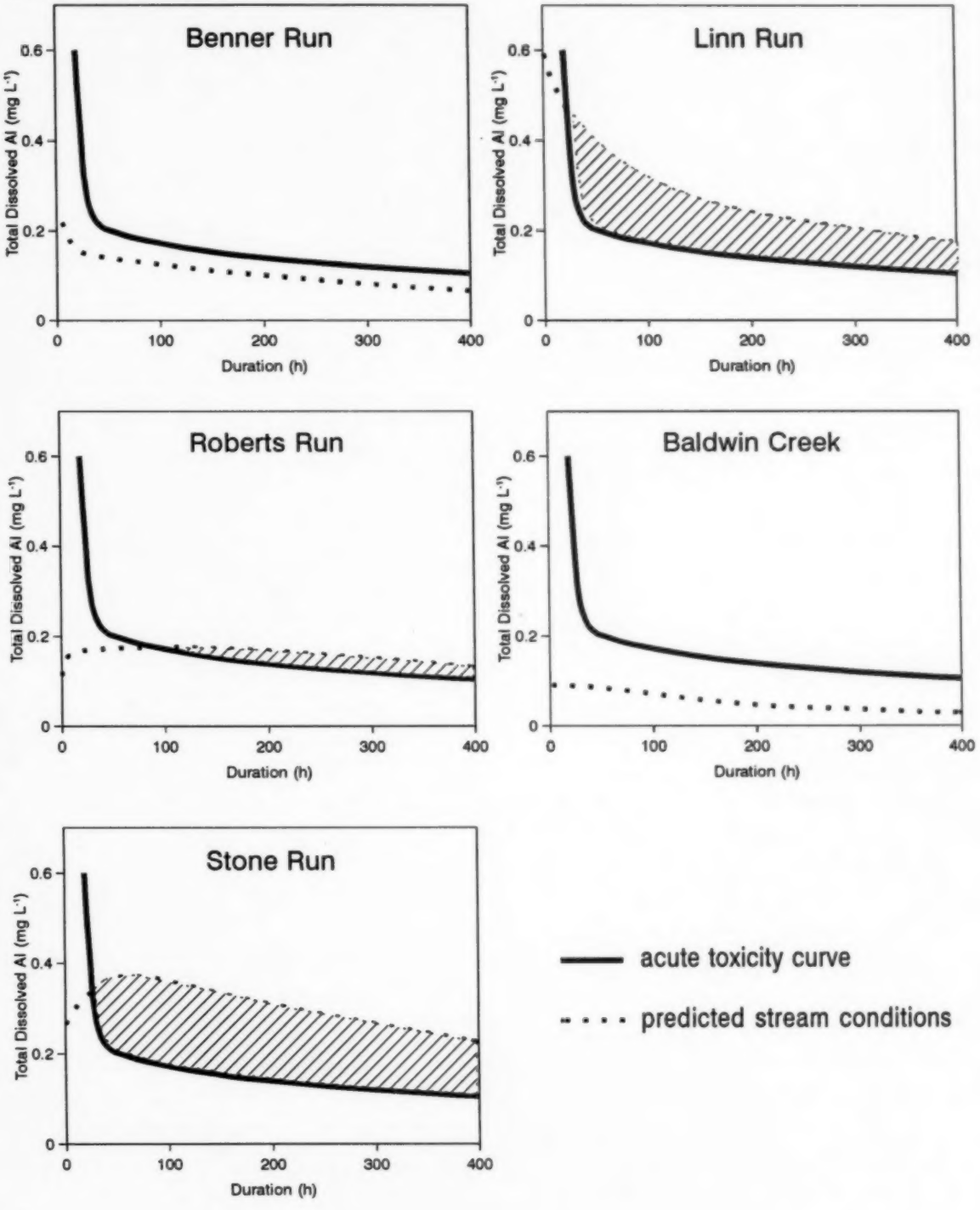


Table 2. Predicted occurrence of lethal episodes during the 19-month study period on each study stream in relation to age one-year and older brook trout population densities.

Stream	Percentage of study period with lethal Al_{TD} episodes (%)	Number of lethal episodes during the study period	Mean density of age 1-year and older brook trout[a] (no.·0.1 ha^{-1})
Baldwin Creek	0	0	95*a*
Benner Run	0	0	70*ab*
Stone Run	46	9	60*b*
Roberts Run	28	6	41*b*
Linn Run	22	11	5*c*

[a]Mean population densities were calculated from 10 electrofishing surveys conducted during 1988–1990. Means with different letters are statistically different ($p < 0.05$) using the Tukey multiple means comparison method.

for 24–48 h or Al_{TD} from 100–200 $\mu g \cdot L^{-1}$ for 48–400 h may be lethal to caged brook trout. Various combinations of Al_{TD} concentrations and exposure durations defining the toxicity curve are shown in Fig. 4 and are used for later computations. These results agree with previous studies, which have suggested that aluminum concentrations in excess of 100–200 $\mu g \cdot L^{-1}$ were necessary to produce brook trout mortality (Baker et al. 1990).

Episodic Al_{TD} toxicity in streams

Al_{TD}–duration curves were derived from the episodic flow–duration curves (Fig. 2) and the Al_{TD}–flow relationships (Table 1) for each stream and are shown as dotted lines in Fig. 5. The toxicity curve for brook trout (from Fig. 4) is plotted against each Al_{TD}-duration curve to show regions where lethal episodes occurred (hatched areas in Fig. 5). The Al_{TD}–duration curves were always below the toxicity curve on the two reference streams (Benner Run and Baldwin Creek); thus, episodes of sufficient Al_{TD} and duration to cause brook trout mortality never occurred on these streams during the study period (Fig. 5). Episodes with varying degrees of severity did occur on Linn, Roberts, and Stone runs (Fig. 5). Episodes on Roberts Run only slightly exceeded the toxicity curve during longer duration episodes (>100 h). The plateau or decline in Al_{TD} concentrations at very high stream flows on Stone and Roberts runs created Al_{TD}–duration curves where greater toxicity is produced by longer duration moderate flows than by shorter duration peak flow periods as in Linn Run (Fig. 5).

These results agree with Gagen et al. (1993) who showed that significant mortality of caged wild brook trout occurred in Linn, Stone, and Roberts runs but not in Benner Run or Baldwin Creek. The results also appear to corroborate the work of Gagen et al. (1994) that demonstrated that there was greater downstream movement of radio-tagged, free-ranging brook trout in conjunction with episodes on Linn and Stone runs compared with Baldwin Creek and Benner Run.

The lethal conditions produced by the overlap of episodic flow–durations and Al_{TD} concentrations (hatched areas, Fig. 5) were studied in detail to determine the number of discrete lethal episodes and the percentage of the total study period occupied by these episodes (Table 2). Of the three streams with lethal episodes, Linn Run experienced the most lethal episodes (11) compared with Stone Run (9) and Roberts Run (6). The percentage of the 19-month study period occupied by lethal episodes varied from 22% on Linn Run to 46 percent on Stone Run (Table 2).

Trout population response

Population densities of native brook trout were estimated to determine if the occurrence of lethal episodes was affecting wild brook trout populations in the five streams (Table 2). Significantly larger mean population densities were observed in the two reference streams where lethal episodes were not predicted to have occurred (Table 2). Lower population densities existed in the three streams with predicted lethal episodes, especially in Linn Run where an average of only five adult brook trout per 0.1 ha was found. Previous research has shown that the study section of Linn Run does not support a reproducing trout population, but a remnant population is supported by immigration from several alkaline tributaries (Carline et al. 1994). The periodic occurrence of lethal episodes on Roberts Run yielded a low population density of 41 trout per 0.1 ha.

The presence of a modest brook trout population in Stone Run (Table 2) despite the predicted occurrence of numerous lethal episodes and high mortality of caged wild brook trout (Gagen et al. 1993) was surprising. There are several possible hypotheses to explain this occurrence including (*i*) cage-induced mortality of fish during the caged fish experiments or (*ii*) migration of fish into the study section. The lack of any significant cage-induced mortality in the two reference streams suggests that there was not an inherent design-induced cage effect in this study (Gagen et al. 1993). Migration is known to occur out of the study section on Stone Run during episodes (Gagen et al. 1994). Our electrofishing surveys, which were generally conducted at lower flows to maximize catch efficiencies, may be measuring a largely transient population in this stream. Carline et al. (1994) hypothesized that Stone Run population densities were unusually high because there is a substantial area downstream of the study segment from which immigration may occur. Periodic sampling downstream of the Stone Run study section did

reveal lower Al_{TD} concentrations compared with the study section.

This paper presents a technique for integrating stream flow, concentration, and duration effects to determine the occurrence of lethal aluminum episodes in five headwater streams. Determining the biological effects of episodic acidification compared to chronic acidification is difficult owing to the transient and complex nature of stream and lake hydrochemical changes. The frequency of episodic occurrences, especially in humid climates, makes incorporation of aquatic effects from this phenomenon necessary. On streams such as Linn Run, where stream chemistry conditions are favorable for brook trout during low stream flows, high Al_{TD} concentrations during acidic episodes help to explain the depauperate, nonreproducing trout population. With fundamental knowledge of Al_{TD}–flow relationships and continuous flow records, impacts of episodic aluminum toxicity can be quantified. Although the measured fish population densities generally conformed to estimated episodic occurrence of toxic Al_{TD} conditions in the five study streams, rapid upstream migration of trout from stream areas of more favorable water quality probably allowed recolonization of some of the study sections.

The general episodic flow–duration curve procedure described in this paper could be applied to other pollutants and target organisms. Episodic flow–duration curves as in Fig. 2 can be developed for many streams because flow data (at least average daily flows) are commonly available. Other stream pollutants could be used as long as the concentration is correlated with stream flow and the toxicity to a target organism depends on both concentration and duration of exposure. Numerous studies have hypothesized or reported increases in other biologically relevant pollutants during acidic episodes including H^+ (Baker et al. 1990; Wigington et al. 1990), manganese (Borg 1986; Lindberg and Turner 1988), and mercury (Lindqvist 1991; Driscoll et al. 1994). Other studies have reported positive correlations between stream flow and concentrations of heavy metals, polychorinated biphenyls, and some pesticides (Granier et al. 1990; Bannerman et al. 1993) in urban runoff. Increased concentrations of many pesticides have also been reported with increased stream flow especially during spring and early summer storms (Norris 1969; Goolsby et al. 1991; Thurman et al. 1991; Squillace and Thurman 1992; Coupe et al. 1993; Ebise et al. 1993). Although it is apparent that many pollutants could increase with stream flow, relationships between concentration and flow for a particular stream and pollutant would likely be difficult to obtain and would probably require on-site data collection until more general relationships can be developed.

The final requirement for application of the proposed method would be some estimate of the concentration–duration toxicity curve (Fig. 4) for the pollutant and target organism of choice. In addition to Al_{TD}, exposure–duration effects on pollutant toxicity have been reported for a variety of pollutants including H^+ (Curtis et al. 1989), various metals (McKee and Wolf 1963; McKim et al. 1976; Nriagu 1979, 1980*a*, 1980*b*, 1980*c*; Moore and Ramamoorthy 1984), and pesticides (Alabaster and Lloyd 1982; Murty 1986; Grande et al. 1994). With some research into the available literature, an approximate concentration–duration toxicity curve as in Fig. 4 could be constructed for many pollutants and target organisms based primarily on controlled laboratory bioassays.

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Temporal variability in the chemical characteristics along the Rivière de l'Achigan: How many samples are necessary to describe stream chemistry?[1]

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Abstract: We examined the temporal variability of several chemical characteristics at 12 different sites in a river system of the Laurentian region of Quebec. Mean–variance relationships (log–log) differed considerably among chemical variables with slopes ranging from about 1.3 to over 5. Guidelines and equations are given to help decide how many samples should be taken to obtain a mean value with an error of 20% or less for the different chemical variables. Relative temporal variabilities (coefficients of variation) were highest for nutrients and lowest for pH, alkalinity, and conductivity. Data from the same sites but for different years showed no systematic differences and similarly mean–variance relationships for TP and TDP obtained from a different region were not significantly different suggesting a wider applicability to our results. We also show that although the temporal variability of chlorophyll in streams is similar to that found in lakes, the same does not apply to phosphorus, which is about two to three times more variable in streams.

Résumé : Nous avons examiné la variabilité temporelle de plusieurs variables chimiques sur 12 sites d'une rivière des Laurentides, Québec. Les relations moyenne–variance (log–log) diffèrent considérablement entre variables chimiques avec des pentes variant entre 1.3 et plus de 5. Certaines lignes directives et équations sont données de façon à prédéterminer combien d'échantillons sont nécessaires afin d'obtenir une valeur moyenne avec 20% d'erreur ou moins. Les variabilités temporelles relatives (coefficients de variation) étaient les plus élevées pour les éléments nutritifs et les moins élevées pour le pH, l'alcalinité et la conductivité. Les données provenant de mêmes sites mais de différentes années n'ont pas montré de différences systématiques et, de façon similaire, les relations moyenne–variance pour le PT et le PTD obtenues d'une autre région n'étaient pas significativement différentes, suggérant une plus grande applicabilité à nos résultats. Nous démontrons également que malgré la variabilité temporelle du phosphore soit deux à trois fois plus grande en rivières qu'en lacs, la variabilité temporelle de la chlorophylle était, elle, environ la même dans les deux milieux.

Introduction

Streams are notoriously variable. Following storms, stream runoff increases and can alter the water chemistry by severalfold within a few hours (Meyer and Likens 1979; Munn and Prepas 1986; Prairie and Kalff 1988*b*). Despite this potentially high temporal variation, in most ecological studies the chemical variables are collected at the time of the biological sampling and because the sorting, counting, and identification of the biological samples are very time consuming, sampling frequency is necessarily often low. It is questionable whether such episodic sampling can accurately represent the stream chemical characteristics. Methods exist to help in the design of sampling programs. In particular, mean–variance relationships have been widely used to establish the number of samples necessary to achieve a desired precision level in the estimate of various aquatic populations (e.g., Downing 1979; Morin 1985; Morin and Cattaneo 1992). A similar approach has been applied both at a regional and global scale to establish optimal sampling frequency for chlorophyll (Knowlton et al. 1984; Marshall et al. 1988; Hanna and Peters 1991) and phosphorus in lakes (France and Peters 1992). To our

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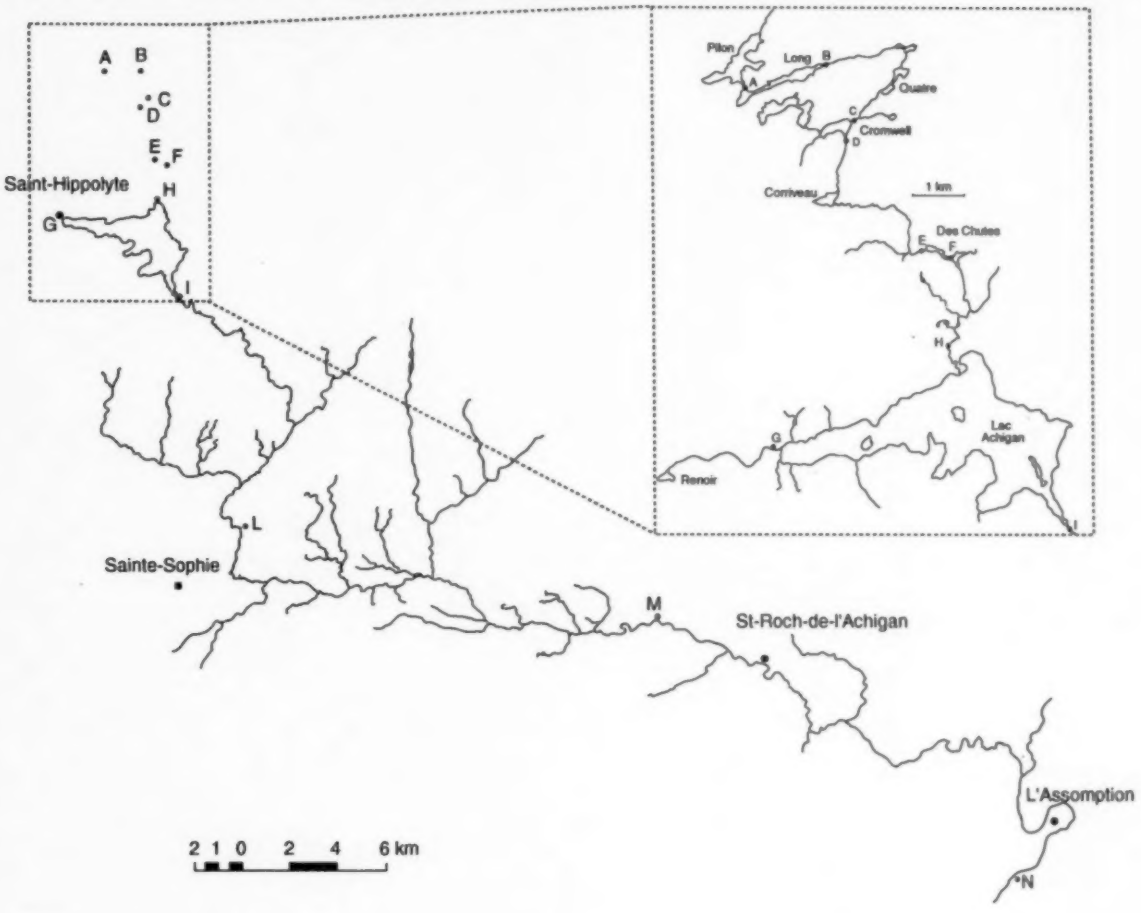
Fig. 1. Map showing the location of the sampling sites. General characteristics are given in Table 1.



knowledge, however, this approach has been attempted only once in streams for some headwater sites in the Eastern Townships of Quebec (Prairie and Kalff 1988*a*, 1988*b*). As their study dealt only with phosphorus, it is unknown whether other important nutrients or chemical characteristics would require a different sampling effort to reach the same level of precision.

In general, stream characteristics change strongly from the headwaters to higher order segments (Vannote et al. 1980). Sites located close to the source may have more constant chemical concentrations while further downstream drainage from larger and more diversified catchment zones could result in stronger temporal variations. The presence of lakes along the stream can also affect temporal variability by acting as a buffer against extreme changes in water discharge.

In this study, we measured the temporal variation in stream chemistry by sampling weekly 12 stations along the Rivière de l'Achigan over one summer. The stations were located at different distances from the source and downstream from a series of lakes to test the potential influence of both stream size and the presence of lakes on

Table 1. Order, distance from headwater (DH), and distance downstream a lake (DL) for the 12 stations. The lakes upstream the stations are listed with their surface area (ha) in brackets.

Station	Order	DH (m)	DL (m)	Lake (ha)
A	1	40	40	Pilon (31)
B	2	1460	10	Long (18)
C	2	4860	400	Quatre (7)
D	2	5460	5	Cromwell (9)
E	2	9020	2200	Corriveau (6)
F	2	9570	150	Des Chutes (3)
G	1	2610	2610	Renoir (3)
H	3	11620	1780	Des Chutes (3)
I	3	15620	5	Achigan (529)
L	4	19570	1350	Achigan (529)
M	4	41970	23750	Achigan (529)
N	5	75390	57170	Achigan (529)

Table 2. Statistics of the mean–variance relationships expressed as power functions $Var = \alpha X^{\beta}$ for the different chemical variables. N_{pred} is the power function equation allowing the prediction of the number of samples necessary to obtain a mean with an error of 20% or less. Color is in Abs_{440} units, conductivity in µS, alkalinity in µequiv.·L^{-1}, and all other concentrations are in µg·L^{-1}.

Chemical variable	α	β	r^2	N_{pred}	Range
Si	5.50	1.37	0.54	$137.5\ [Si]^{-0.63}$	1256–7760
TN	2.3×10^{-6}	3.75	0.81	$5.75 \times 10^{-5}\ [TN]^{1.75}$	291–1037
TDN	2.12×10^{-5}	3.39	0.91	$5.3 \times 10^{-4}\ [TDN]^{1.39}$	217–796
NO_3	0.15	2.09	0.86	$3.75\ [NO_3]^{-0.09}$	20–596
TP	0.51	1.88	0.86	$12.75\ [TP]^{-0.12}$	4.3–71
TDP	0.52	1.83	0.93	$13\ [TDP]^{-0.15}$	1.4–40
Chlorophyll *a* (Chl *a*)	0.21	2.13	0.54	$5.25\ [Chl\ a]^{0.13}$	1–3.7
Colour	9.97	5.17	0.88	$249\ [Color]^{3.17}$	0.046–0.318
pH	4.87×10^{-7}	5.81	0.17[a]	$1.22 \times 10^{-5}\ [pH]^{3.81}$	6.43–7.45
Conductivity (Cond.)	7.9×10^{-7}	4.75	0.59	$1.98 \times 10^{-5}\ [Cond.]^{2.75}$	22–73
Alkalinity (Alk.)	0.019	2.14	0.85	$0.475\ [Alk.]^{0.14}$	127–814

[a]Not significant ($p > 0.05$).

Table 3. Calculated number of replicates necessary to obtain a mean with an error of 20% or less for the minimum and maximum concentrations of the different chemical parameters observed in our study.

Chemical parameter	$N_{min\ [\ ]}$	$N_{max\ [\ ]}$
Si	2	<1
TN	1	11
TDN	1	6
NO_3	5	7
TP	11	8
TDP	12	8
Chlorophyll *a*	5	6
Colour	<1	7
pH	<1	<1
Conductivity	<1	3
Alkalinity	<1	1

the temporal variability of chemical characteristics. In all, we collected information on 11 chemical variables. The principal goal was to provide guidelines on the number of samples required over a season to characterize reliably the stream chemistry with a given precision level. The chemical variables we monitored were chosen because they are most commonly used as correlates in studies of the distribution and abundance of stream organisms.

Methods

Study site

Rivière de l'Achigan, a low-gradient tributary of the St. Lawrence River is located in the Lower Laurentians, Québec (45°59′N, 74°01′W). It extends 75 km from the headwater Lac Pilon to the confluence with Rivière l'Assomption and links a chain of small lakes. Its hydrological regime is characterized by a spring peak and low but permanent summer flows. We choose 12 stations to represent a large range of distance from headwater and upstream lakes (Fig. 1, Table 1). Two stations are first order draining headwater lakes (A and G) while the most downstream stations are of higher order. The watershed is forested and pristine in the upper section until Lac Des Chutes; some cottages are present in the middle section, but agriculture and animal farms enrich the most downstream stations (M and N). To test the potential effect of lakes, some stations were located immediately upstream or downstream from four lakes (Lac Long, stations A and B; Lac Cromwell, stations C and D; Lac Des Chutes, stations E and F; lac Achigan, stations H and I). Boulders are the dominant substratum in the upper sections but fine substratum become increasingly common downstream. The bedrock is Precambrian anorthosite (McGerrigle 1976).

Sampling and chemical analysis

The stations were visited weekly for 14 consecutive weeks (14 May – 14 August 1993) starting after the spring runoff had subsided. Sampling was regularly spaced with no attempt to follow or avoid particular meteorological events. Our sampling protocol can, therefore, be considered random with respect to rain events. Water was collected by wading in the eight upstream stations, while in the remaining four stations, a sampling bucket was lowered from a bridge.

Sestonic chlorophyll was estimated by filtering 1 L of water through Gelman glass fiber filters (type AE). These filters were kept frozen until analysis, when the chlorophyll was extracted in 96% ethanol and measured spectrophotometrically (Bergman and Peters 1980).

Water colour was determined by measuring the absorbance at 440 nm (Abs_{440}) in a 10-cm path length cuvette of water samples filtered through Gelman glass fiber (type AE) filters. These can be converted to standard gilvin colour units (g) using the following equation:

Table 4. Mean and coefficients of variation (CV, %) of all chemical parameters studied at each station. Color is in Abs_{440} units, conductivity in μS, alkalinity in μequiv.·L^{-1}, and all other concentrations are in μg·L^{-1}.

Station		Si	TN	TDN	NO_3	TP	TDP	Chlorophyll *a*	Color	Conductivity	pH	Alkalinity
A	Mean	2095	291	217	52	5.7	2.5	1.1	0.046	49	6.8	179
	CV	22	25	16	68	55	65	31	18	4	2	14
B	Mean	2331	393	293	22	8.0	3.9	1.8	0.135	35	6.6	148
	CV	25	26	28	55	51	55	35	8	7	2	27
C	Mean	1490	477	360	76	12.4	7.4	2.5	0.183	22	6.4	127
	CV	28	26	32	46	36	41	62	10	10	2	12
D	Mean	1256	419	298	21	10.1	4.6	3.7	0.136	22	6.5	142
	CV	23	28	32	73	46	64	34	9	8	4	29
E	Mean	3539	452	382	114	15.0	10.3	1.5	0.136	28	6.8	158
	CV	12	41	33	41	70	64	113	18	12	3	17
F	Mean	3591	440	359	98	15.4	6.8	2.6	0.144	31	6.8	180
	CV	12	37	29	28	111	115	128	24	11	3	19
G	Mean	7760	501	465	105	24.5	14.4	1.0	0.241	51	7.3	502
	CV	12	25	27	41	79	46	34	23	32	3	24
H	Mean	3816	413	365	126	10.8	5.6	1.1	0.153	43	7.0	237
	CV	23	23	26	33	81	60	51	14	12	2	19
I	Mean	4995	308	242	113	4.3	1.4	1.7	0.061	54	7.0	248
	CV	10	20	18	41	75	78	32	9	4	3	29
L	Mean	5083	587	369	129	11.6	4.8	1.5	0.172	72	7.1	364
	CV	15	93	23	26	71	66	48	36	33	3	19
M	Mean	5595	883	748	506	52.4	29.4	3.7	0.318	64	7.3	782
	CV	30	39	33	68	51	65	42	32	75	2	15
N	Mean	5821	1037	796	597	71.1	40.1	3.5	0.269	71	7.5	814
	CV	23	72	63	101	47	55	36	48	99	5	27

$$g_{440}(m^{-1}) = \frac{2.303 Abs_{440}}{r}$$

where r is the cell path length (m) (Kirk 1983). Empirical equations to convert them to other color units are given in Cuthbert and del Giorgio (1992).

Unfiltered samples were used to determine total phosphorus and total nitrogen while total dissolved phosphorus (TDP), total dissolved nitrogen (TDN), nitrates, and silicate were measured on samples filtered on site through 0.45-μm membrane syringe filters. All samples were taken in triplicate. Total and total dissolved nitrogen samples were measured as nitrates after alkaline persulfate digestion (0.075 g $K_2S_2O_8$ and 0.015 g NaOH per 10-mL sample) and autoclaving for 90 minutes (see Ameel et al. 1993). Nitrate and digested nitrogen samples were analyzed by the cadmium reduction method (Alpkem method A303-S170-33, based on U.S. EPA Method 353.2). Total phosphorus and total dissolved phosphorus concentrations were determined using the molydenum blue method after persulfate digestion and autoclaving and the digested samples analyzed as ortho-phosphates (Alpkem Method A303-S200, U.S. EPA Method 365.3). Silicate concentrations were determined also with the molybdenum blue method (Alpkem method A303-S220). All chemical analyses were performed on an Alpkem RFA300 autoanalyzer. Inter-replicate variability (standard deviation) for midrange concentrations varied between 3 and 7% of the mean depending on the chemical variable.

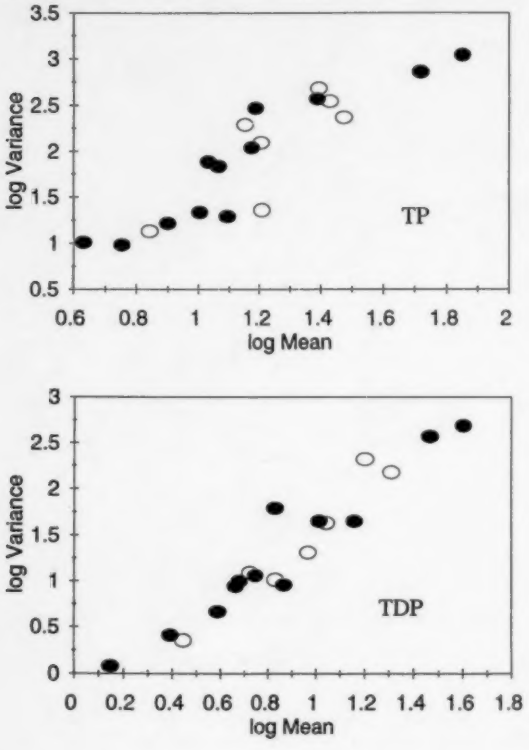
All statistical analyses were performed using SAS (Statistical Analysis System), version 6.08. In cases where the results of many correlation analyses were examined simultaneously, we applied a Bonferonni correction to ensure an overall 0.05 probability level for statistical significance. Coefficients of the mean–variance relationships were estimated using ordinary least-squares with the following model: $\log_{10} \text{Var} = \log_{10} \alpha + \beta \log_{10} \text{mean}$. The number of samples necessary to obtain a given level of precision was calculated following standard techniques (e.g., Downing 1979; Morin 1985).

Results

General mean–variance relationships and sampling frequency

Temporal variability of most of the chemical variables increased steadily with mean concentration. Plots of the temporal variance as a function of the mean (on a log–log scale) showed strong relationships for all variables, except pH. However, the rate (slope of log–log relationship) at which variability increased with the mean varied greatly among the variables examined, suggesting that sampling protocols should be designed specifically for the variables of interest. As a standard of comparison, a slope of 2 corresponds to a constant temporal variability relative to the mean, i.e., a constant coefficient of variation (CV). Slopes greater than 2 imply increasing CV with the increasing mean (and hence increased sampling effort required) while slopes smaller than 2 imply the reverse.

Fig. 2. Scatterplots of the mean–variance relationships for TP and TDP combining data from this study (solid circles) and data from the Lake Memphremagog area (open circles). Log_{10} mean values are in $\mu g \cdot L^{-1}$.



Some features of the mean–variance relationships and the range of mean values over which they apply (Table 2) are noteworthy. First, some of the differences among the chemicals were systematic and related to chemical groups. Total nitrogen (TN) and total dissolved nitrogen (TDN) had high and similar slopes (3.39 and 3.75, respectively). In the same way, total phosphorus (TP) and total dissolved phosphorus (TDP) had almost identical mean–variance relationships with slopes around 1.85. Second, some of the chemical constituents had remarkably different high slopes, ranging from 1.37 to over 5. For example, while a 10-fold increase in water color is accompanied by an increase of more than a 100 000 times increase in its temporal variance (slope greater than 5), the same augmentation in silica concentration only increases the temporal variance by 23-fold (slope less than 1.4, Table 2).

Table 2 also provides the equations allowing one to calculate the number of random samples necessary to obtain an estimate of the mean with a standard error of the mean ($CV_{mean} = SE_{mean}$/mean) of 20% or less. As guidelines, we solved these equations for the minimum and maximum concentrations found in our study sites for each variable (Table 3). For some variables, the calculated number of samples required was nearly independent of the concentration (within the range of concentrations we observed) while in our most variable case, total nitrogen, the number of necessary samples varied from 1 for a TN-poor site to 11 for a TN-rich site. That TP and TDP require more sampling effort at the lowest concentrations can be attributed to the fact that at these concentrations, temporal variance approaches inter-replicate variability (the minimum possible variability), which relative to the mean value (around 1–2 $\mu g \cdot L^{-1}$), requires proportionately more replicates for a 20% precision level.

These equations can be used directly to optimize future sampling programs when pilot surveys can be done to obtain a rough estimate of the mean. However, when such pilot surveys are not available or impracticable, our results suggest that unless the average TN concentration exceeds 900 $\mu g \cdot L^{-1}$, eight samples appear sufficient to characterize reliably the average stream chemistry (for the variables analyzed).

Influence of other factors on the temporal variability

Mean–variance relationships not only provide guidelines for the planning of sampling efforts but also for the magnitude of temporal variability that one should consider normal for a given site (i.e., a given mean). We, therefore, examined the influence of other factors on temporal variability by testing whether they were correlated with the residuals (observed minus predicted) of the mean–variance relationships. The factors we examined were the distance from the headwater source (DH) and the distance from the nearest upstream lake (DL).

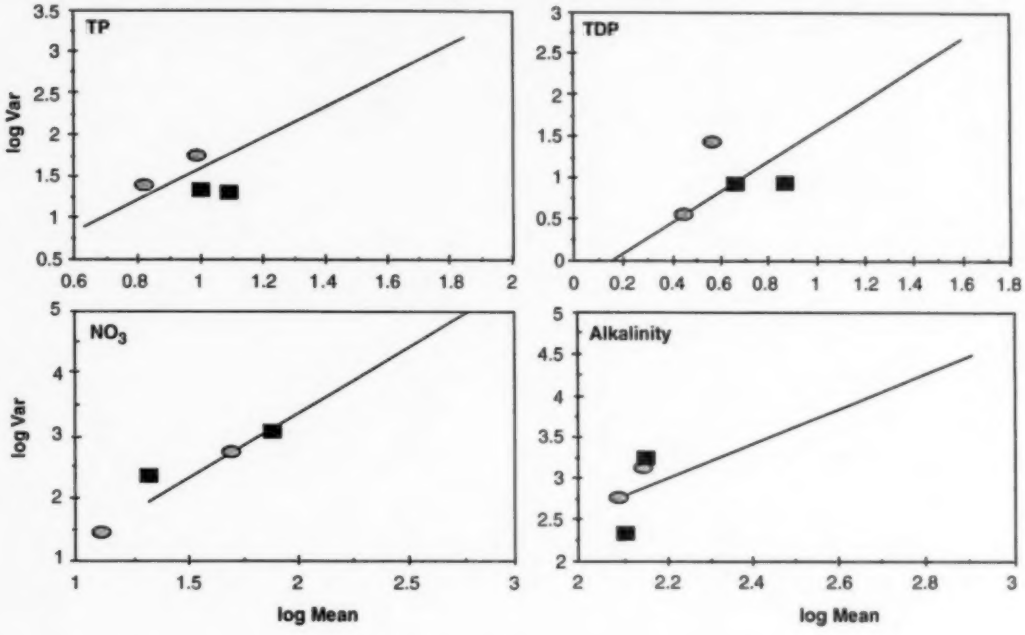
We found to our surprise that, although the distance from the headwaters and the distance from the lake were related to the mean concentration of several variables (TN, TDN, NO_3, and colour increased with increasing distance from headwater site, see Table 4), we obtained no significant correlations between either DH or DL and the residuals of any of the 12 mean–variance relationships. This demonstrates that stream sites close to the headwaters are not less temporally variable (relative to their mean) than sites further downstream. Similarly, when we looked for a damping effect of lakes by examining whether the residuals were systematically below the mean–variance lines for sites located immediately downstream of lakes (sites B, D, F, and I), we found no consistent pattern.

As a further attempt to identify factors that might be responsible for particularly low or high variance, we tested whether sites that had a low mean–variance residual for one chemical variable also had low residuals for the other variables. If this were the case, it would identify the sites with low variance and, therefore, guide us in defining the particularities of that site. To do so, we simply computed a correlation matrix of all the residuals. Here again, we were not able to detect any significant correlations.

Comparison with other regions and other years

The only other region for which we had data to calculate mean–variance relationships is the Eastern Township region of Quebec for which the dynamics of phosphorus had been studied in some detail (Prairie and Kalff 1988*a*, 1988*b*). The Eastern Townships substantially differ from the Laurentian region in terms of geology (off and on the

Fig. 3. Comparison of the temporal variability of TP, TDP, NO_3, and alkalinity between two sampling years at two stations (C and D). Circles are for 1989 while squares are for 1993. Solid lines are the mean–variance relationships developed from the data of this study (equations in Table 2).



Canadian shield, respectively). The comparison could only be done for total phosphorus and total dissolved phosphorus (Fig. 2). In both cases, an analysis of covariance (saturated model) revealed no significant differences between regions. We suggest that the possibility of a greater generality to our results than what might have been expected on the basis of the restricted area of our study merits further investigation.

Similarly, we were able to assess how the temporal variability over the summer varied from year to year because one of us (Y.T.P.) collected stream chemistry data in 1989 at stations C and D. However, because mean concentrations also varied between years (and the associated changes in temporal variance), annual variance values could not be compared directly. Instead, we examined how the 2 years fitted around the mean–variance relationships developed above for the chemical variables common to both studies: TP, TDP, NO_3 and alkalinity (Fig. 3). We found no systematic deviation from the mean trend lines for any of the variables.

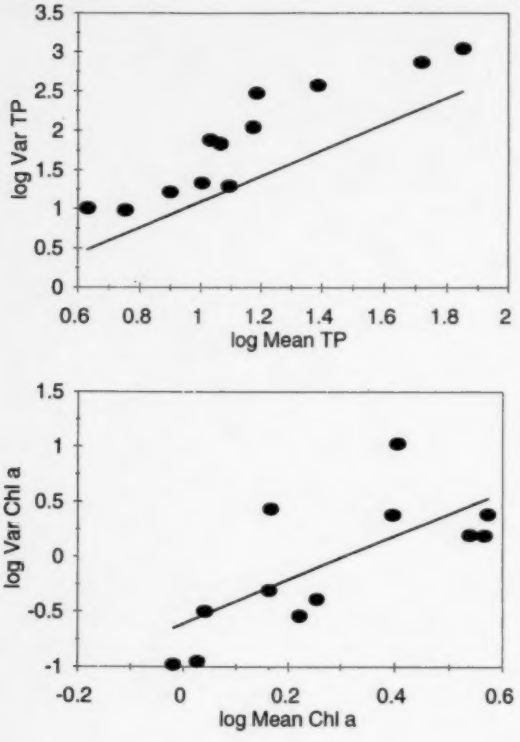
Comparison with lakes

Temporal mean–variance relationships have been developed for lakes for both chlorophyll *a* (Knowlton et al. 1984; Marshall et al. 1988) and total phosphorus (France and Peters 1992) concentrations. We compared the temporal variability for these two variables between lakes and streams and found that, while the mean–variance functions were nearly identical for chlorophyll *a* (Fig. 4), phosphorus was about two to three times more temporally variable (for a given mean concentration) in streams than in lakes (Fig. 4). Such a difference clearly indicates that greater sampling effort is required to characterize reliably the mean phosphorus concentration of streams than of lakes.

Discussion

There is a widespread belief that, particularly for small streams and rivers, water chemistry is too variable to quantify with a reasonable number of samples. Our study shows that, depending on the particular chemical variable and concentration, 1–12 samples are needed to obtain an estimate of the summer mean with a 20% error or less. Such differences in the effort required for different chemical variables had also been noted in lakes (Harris 1987). As a rough rule however, we suggest that about eight samples are probably sufficient to estimate reliably the mean of most variables. This is not an unrealistic number even for ecological studies requiring lengthy sample procedures. However, particular attention should be given when applying these guidelines that the expected concentrations are within the ranges observed in our study (Table 2). This is especially true of chemical parameters having high mean–variance slopes (TN, TDN, colour, pH, and conductivity). Care should be also taken not to interpret these summer temporal variability functions as measures of the annual variability the variables are likely to experience. For example, in several Canadian streams, drastic episodes of depressed alkalinity and pH are observed during snowmelt (Tranter et al. 1994), a time period not considered in our

Fig. 4. Comparison of the mean–variance relationships for chlorophyll and phosphorus between lakes and streams. Solid circles are observations from this study (streams) while the solid line repersents the mean–variance relationship developed for lakes by France and Peters (1992).



study. In addition, not all chemical variables are likely to exhibit the same seasonal pattern in temporal variability.

Our study also suggests that the mean–variance relationships found could be valid beyond this particular region or this particular summer as no differences were found with the Eastern Townships data and for data from different years. Thus, the applicability of the proposed sampling guidelines should be tested when intended for vastly different geographies, climates, or river morphologies. For instance, data extracted from a study of Biggs and Close (1989) in a series of alpine fed New Zealand streams, where rapid flow events are common, show much higher temporal variability (CV of total phosphorus up to 276%).

A direct comparison of temporal variability among chemical variables cannot be inferred from the mean–variance relationships alone but an examination of the coefficients of variation at each station (Table 4) suggests that the order of temporal variability (as CV) can be generalized as follows TP, TDP, chlorophyll a > NO_3 > TN, TDN > Si, alkalinity > conductivity, colour > pH. Interestingly, the order of the nutrients within the above sequence corresponds also to their believed importance as limiting resources (P > N > Si) in this P-limited system (20 < TN : TP < 160), supporting the notion that summer temporal variability in nutrient chemistry is largely biologically driven. Differences in variability among different chemical characteristics have been observed also in lakes. In a study of daily chemical variations in Lake Ontario, Harris (1987) found that phosphorus was the most variable element. Higher temporal variability of nutrients compared with other ions has been observed in a study of the baseflow chemistry of 96 New Zealand streams (Close and Davies-Colley 1990). Following fast flood in a Sonoran desert stream (Fisher and Grimm 1982), nutrient concentrations changed sixfold while conductivity increased only slightly (<20%); in this N-limited system, nitrogen was the most variable element. Hence, the generality of the observed correspondence between limitation and variability should be explored further as it may result in a useful rule.

We were not able to detect any influence of the distance from the headwaters (DH, a measure of stream size) suggesting that the generally accepted but rarely tested notion that large rivers are less variable than small ones should be reconsidered, at least for streams of order 5 and lower. Similarly, we found no effect of distance from lakes (DL) casting doubts again about the generality of the buffering effects of lakes. Nevertheless, other factors likely to influence temporal variability such as land use practice or discharge regime could not be addressed in a study of a single river system. However, the seasonal variability in phosphorus was greater in our streams than in lakes (France and Peters 1992) over a wide range of concentrations (Fig. 4). This probably reflects the dramatic influence of water discharge on stream phosphorus concentration whether dissolved or particulate (Rigler 1979; Meyer and Likens 1979; Prairie and Kalff 1988*a*, 1988*b*) in comparison to its influence on lake P concentration. That we did not observe this damping effect in our lake outlet stations is probably because most of our lakes are very small and rapidly flushing.

The mean–variance relationship for chlorophyll is, however, the same in both streams and lakes and this is more unexpected given that the phenology of chlorophyll development is not believed to be driven by the same factors in the two environments. We offer two possible explanations. First, stream sestonic chlorophyll is largely of lake origin in these systems although it is strongly reduced by filtering invertebrates particularly near lake outlets. Nevertheless, the temporal variability observed at a given station should still parallel that of the input signal, i.e., the lake chlorophyll. We suggest that this similarity between lakes and streams may well break down in larger rivers where a greater portion of the chlorophyll is autochtonous. A second possibility is that the chlorophyll mean–variance relationship for streams is not as strong as that for lakes (r^2 = 0.54 and 0.90, respectively; France and Peters 1992) thereby reducing our power to discern real differences. Only additional data will resolve this question.

In summary, we have provided guidelines for the design of stream sampling protocols for several chemical variables. Although the mean–variance relationships differed considerably among chemical variables, they were confirmed with data from a different region and sampling year. In our system, the most variable parameters were nutrients, particularly phosphorus. We found no effect of

position relative to upstream lake or from headwater source. The temporal variability of phosphorus was higher in streams than in lakes, but that of chlorophyll was about equal in the two environments.

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Genetic similarity among zebra mussel populations within North America and Europe

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Abstract: The zebra mussel *Dreissena polymorpha* has rapidly established both contiguous and disjunct populations during its spread through eastern North America. If new colonies are founded by small numbers of individuals, populations with markedly different genetic and phenotypic characters could arise (founder effect); this possibility could confound ecological comparisons of populations from different geographic locations. We analyzed genetic differentiation among 18 populations of mussels from the Great Lakes and seven populations from Europe using starch-gel electrophoresis. Analysis of 27 proteins yielded data from 15 polymorphic loci and one monomorphic locus. The data indicated that zebra mussels in North America have the same high genetic variability (H_o = 0.30–0.43) that is found in European populations (H_o = 0.27–0.35) and is typical of molluscs. Little variation appears to have been lost when zebra mussel were transported to North America. Nei's genetic distances between populations were small (0.004–0.028) compared with distances among populations of other mollusc species (0.023–0.184). Like populations from Europe, populations within North America were not highly differentiated, which suggests that founder populations have not been small and (or) frequent genetic mixing has occurred. European populations clustered seperately from North American populations (Nei's distance = 0.058).

Résumé : La moule zébrée (*Dreissena polymorpha*) a rapidement constitué des populations contiguës et isolées, au cours de sa propagation dans l'est de l'Amérique du Nord. Si de nouvelles colonies sont fondées par de petites quantités d'individus, des populations présentant des caractères génétiques et phénotypiques radicalement différentes pourraient surgir (effet fondateur); ce phénomène pourrait confondre les comparaisons écologiques de populations provenant de différents secteurs géographiques. Nous avons analysé la différenciation génétique chez 18 populations de moules des Grands Lacs et de 7 populations d'Europe, par électrophorèse sur gel d'amidon. L'analyse de 27 protéines a fourni des données de 15 sites polymorphes et d'un site monomorphique. Ces données indiquent que la moule zébrée en Amérique du Nord présente la même variabilité génétique (H_o = 0,30–0,43) que les populations européennes (H_o = 0,27–0,35), laquelle est typique des mollusques. La variabilité perdue lors du transport des moules en Amérique du Nord semble minime. Les distances génétiques de Nei entre les populations étaient modestes (0,004–0,028), quand on les compare aux distances constatées chez les populations d'autres espèces de mollusques (0,023–0,184). Comme pour les populations européennes, les populations nord-américaines n'étaient pas hautement différenciées, ce qui laisse à penser que les populations fondatrices n'étaient pas petites ou encore qu'il y a eu de fréquentes combinaisons de populations. Les populations européennes étaient groupées différemment des populations nord-américaines (distances de Nei = 0,058).

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Introduction

Zebra mussel (*Dreissena polymorpha*) populations in Europe are characterized by high variability in number, biomass, production, age structure, and individual size, weight, and color pattern (Stanczykowska 1977; Smirnova et al. 1993). This variability is so striking that it is often difficult to find consistent agreement on the limits of life-history traits such as size and age at reproductive maturity, temperature limits for survival, and (or) growth rates. The populations that have developed in North America differ from their European counterparts in characteristics such as growth

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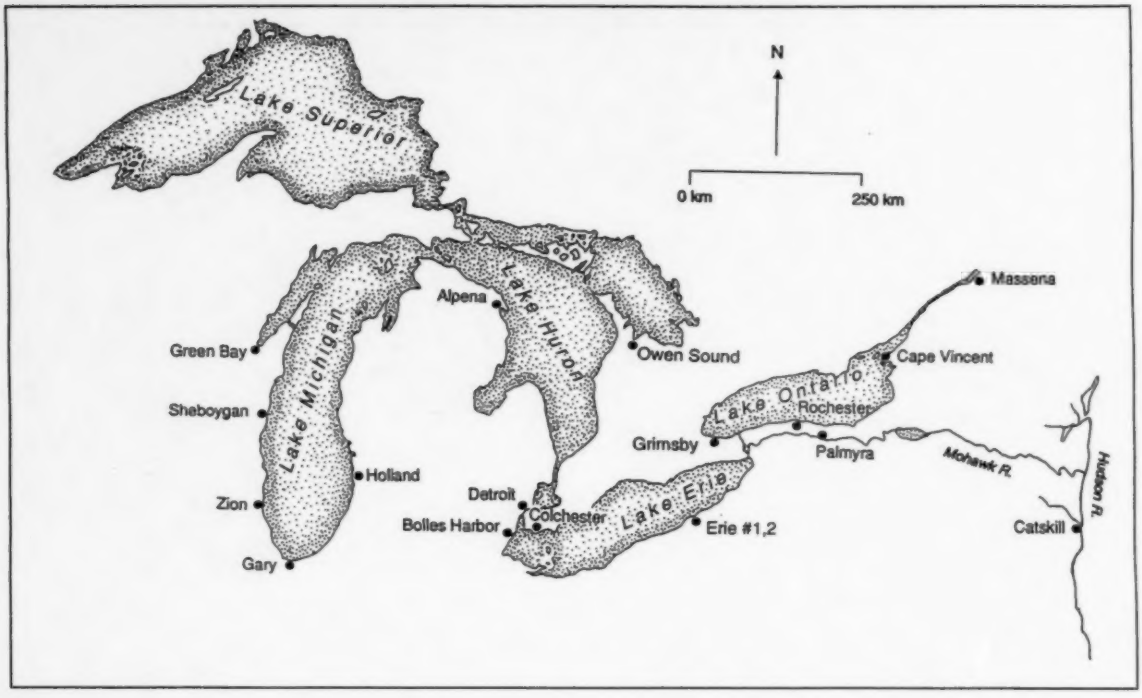
Table 1. Location of zebra mussel populations sampled for this study, with sample size (N) used for genetic analysis. Dates of collection are approximate, based on information from field teams.

Collection date	Abbr.	Sample location	Body of water	N
June 1990	Clair	Detroit, Mich.	Lake St. Clair	45
Oct. 1991	Erie1	Erie, Pa., inside Presque Isle	Lake Erie	80
Oct. 1991	Erie2	Erie, Pa., outside Presque Isle	Lake Erie	40
June 1991	Boll	Bolles Harbor, Ohio	Lake Erie	40
June 1990	Colch	Colchester, Ont.	Lake Erie	43
June 1991	Grim	Grimsby, Ont., at 50-mile Point	Lake Ontario	40
Nov. 1991	Cape	Cape Vincent, N.Y., from Department of Environmental Conservation harbor	Lake Ontario	78
Nov. 1991	Roch	Rochester, N.Y., trawled approximately 2 km offshore	Lake Ontario	40
Nov. 1991	Holl	Holland, Mich., on buoy chain at mouth of L. Macatawa	Lake Michigan	80
Sept. 1991	Green	Green Bay, Wis.	Green Bay	80
Oct. 1991	Zion	Zion, Ill., at Zion-Benton Water Treatment Plant	Lake Michigan	80
Sept. 1991	Sheb	Sheboygan, Wis.	Lake Michigan	80
June 1991	Gary	Gary, Ind., at Union Carbide plant	Lake Michigan	39
July 1991	Owen	Owen Sound, Ont.	Lake Huron	80
July 1991	Alpe	Alpena, Mich.	Lake Huron	40
July 1991	Palm	Palmyra, N.Y.	Erie Canal	39
Sept. 1991	Cats	Catskill, N.Y., near mouth of Catskill Creek	Hudson River	40
Sept. 1991	Mass	Massena, N.Y.	St. Lawrence River	80
Oct. 1991	Polan	Wtoctawek Reservoir, Poland	Wtoctawek Reservoir	80
Oct. 1991	Hung	Budapest, Hungary	River Danube	40
Oct. 1991	Vuren	Vuren, Germany	River Rhine	40
Oct. 1991	Enkh	Enkhuizerzand, Netherlands	Ijsselmeer	40
Oct. 1991	Roer	Roermand, Netherlands	River Meuse	40
Oct. 1991	Peter	St. Petersburg, Russia	Neva River/Lake Ladoga	30
Oct. 1991	Volga	Russia — exact reservoir on Volga River unknown	Volga Reservoir	30

rate, age at reproductive maturity, and temperature tolerances (e.g., Schloesser et al. 1990). In part, these differences among zebra mussel population characteristics can be attributed to differences in their local environments. However, if North American populations of zebra mussels are genetically different from the principal populations studied in Europe, they may have different environmental tolerances. Such differences would exist if a group of founding individuals originated from an unusual population in Europe, or if its genetic character were altered owing to founder effect. Ecological studies tend not to regard genetic influences on the phenotype, i.e., for simplicity, they assume genetic uniformity among populations. However, genetically distinct subpopulations may respond differently to environmental variables as well as to control measures. Such differences could invalidate attempts to make inferences about populations. Zebra mussels have unusually high levels of genetic and morphological variability (Hebert et al. 1989; May and Marsden 1992; Boileau and Hebert 1993). Consequently, there may be large differences in the genetic and, therefore, phenotypic characters among zebra mussel populations. A prime example of this phenomenon was the recognition during this study of a second dreissenid species in the Great Lakes that we termed the quagga mussel (May and Marsden 1992) and that was subsequently identified as *D. bugensis* (Rosenberg and Ludyanskiy 1994; Spidle et al. 1994).

The purpose of this study was to intensively and extensively examine the genetic structure of zebra mussel populations in North America using starch-gel electrophoresis, and to compare these populations with seven populations of mussels collected in Europe. Three alternate hypotheses are proposed. H_1: the mussels represent a single panmictic population across the Great Lakes. In this case, phenotypic differences found between populations will be due to ecological rather than genetic effects. H_2: the mussels have subdivided into genetically distinct subpopulations as they spread across the Great Lakes, owing to founder effect and microevolution. Differences would be due to alterations in allelic frequencies among populations. H_3: some populations are genetically distinct and may represent separate introductions from genetically distinct populations in Europe. Distinct introductions might be detectable by the presence of unique alleles in addition to widely differing allelic frequencies. Under H_2, identification of source populations in Europe is important to understand and predict zebra mussel population behavior in North America. Under either H_2 or H_3, researchers must consider genetic differences when generalizing observations from single populations to the zebra mussels in North America as a whole.

Fig. 1. Locations where zebra mussels were sampled in North America from 1990 to 1991.



Methods

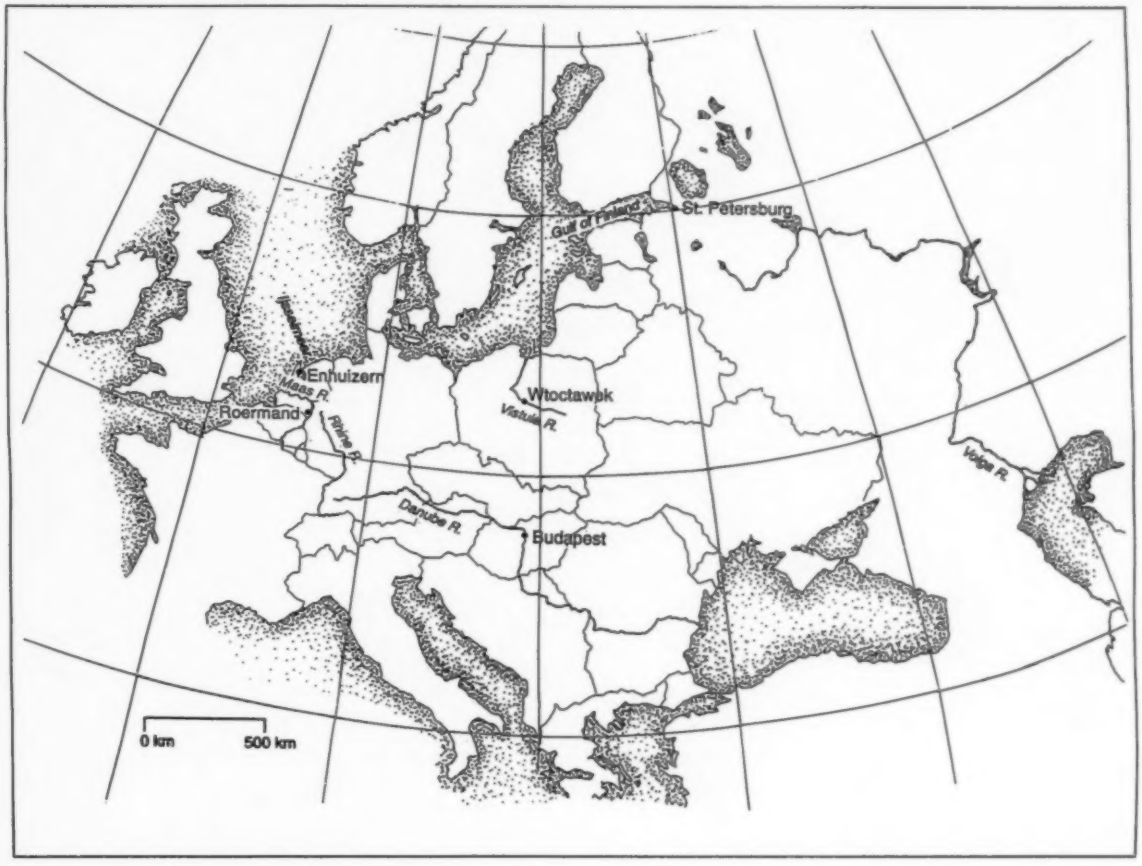
Sample collection

Up to several hundred zebra mussels were collected from a total of 18 locations in lakes Erie, Ontario, Huron, Michigan, and St. Clair, the Hudson and St. Lawrence rivers, and the Erie Canal (Table 1, Fig. 1). When sufficient mussels were available, 80 individuals were sampled from each location for analysis. At several sites insufficient animals could be found, and only 30–40 were available for analysis (Table 1). Larger mussels (>10 mm) were selected so that enough tissue would be available for electrophoretic analysis. All collections were made in the summer of 1990 and the summer and fall of 1991. Seven populations of mussels were sent to us from five European countries (Table 1, Fig. 2). Mussels were frozen on dry ice or in liquid nitrogen within a few hours of collection in the field, shipped to Ithaca, N.Y., and stored at −70°C until processed.

Allozyme analysis

Extracts of whole mussels and one valve from each specimen were homogenized for analysis. Half shells from each mussel were saved for later morphological analysis; these shells will be archived in the Illinois Natural History Survey collections in Champaign, Ill. Twenty-seven proteins were screened for allozyme variability according to methods outlined in May (1992). Genetic and enzyme nomenclature was used according to Shaklee et al. (1990). Aldolase (*ALD**, E.C. 4.1.2.13), aspartate aminotransferase (*AAT**, E.C. 2.6.1.1), glycerol-3-phosphate dehydrogenase (*G3PDH**, E.C. 1.1.1.8) and leucyl-glycyl-glycine peptidase (*PEP-LGG**, E.C. 3.4.11-13) were rejected because they showed limited activity. Adenylate kinase (*AK**, E.C. 2.7.4.3), fructose kinase (*FK**, E.C. 2.7.1.2), glutathione reductase (*GR**, E.C. 1.6.4.2), lactate dehydrogenase (*LDH**, E.C. 1.1.1.27), leucine aminopeptidase (*LAP**, E.C. 3.4.11.1), malic enzyme (*MEP**, E.C. 1.1.1.40), and L-iditol-dehydrogenase (s*IDDH**, E.C. 1.1.1.14) resolved poorly. In many of these cases the number of alleles was too great to allow consistently correct interpretation of the genotypes. Fifteen polymorphic loci for the following proteins were found to be consistently interpretable and were scored for all populations: diaphorase (*DIA**, E.C. 1.6.2.2), esterase (*EST**, E.C. 3.1.1.-), formaldehyde dehydrogenase (*FDH**, E.C. 1.2.1.1), fumarase (*FH**, E.C. 4.2.1.2), glyceraldehyde phosphate dehydrogenase (*GAPDH**, E.C. 1.2.1.12), glucose phosphate isomerase (*GPI**, E.C. 5.3.1.9), isocitrate dehydrogenase (*IDH**, E.C. 1.1.1.42), malate dehydrogenase (*MDH**, E.C. 1.1.1.37), phenyl-alanyl-proline peptidase (*PEP-PAP*, E.C. 3.4.11-13), phosphoglucomutase (*PGM**, E.C. 5.4.2.2), phosphogluconate dehydrogenase (*PGD**, E.C. 1.1.1.44), inorganic pyrophosphatase (*PP**, E.C. 3.6.1.1), and triosephosphate isomerase (*TPI**, E.C. 5.3.1.1). An additional polymorphic locus for aconitase (*AC**, E.C. 4.2.1.3) was scored for all except four populations, and was not included in the genetic distance analyses. One monomorphic locus was found for xanthine dehydrogenase (*XDH**, E.C. 1.2.3.2). Color slides were taken of each gel and kept for archival purposes. Alleles were designated in terms of

Fig. 2. Locations where zebra mussels were sampled in Europe in 1991. Locations of the Vuren and Volga samples are not shown because we are not certain where these samples were taken.



mobility of their homomeric protein product relative to that of the most common allele across all populations at that locus.

Tests for deviation of genotype frequencies from expected Hardy–Weinberg proportions were made using the chi-square contingency test. Genetic differences among samples were compared using the *G*-test, F_{IS} and F_{ST} values (estimates of deviation from Hardy–Weinberg proportions due to differentiation within and among subpopulations), and genetic distance coefficients. Genetic distances (Nei 1972) were calculated using only the 15 polymorphic loci scored, and were subjected to unweighted pair-group method cluster analysis (UPGMA; Sneath and Sokal 1973). Analyses of genetic data were performed using GENES IN POPULATIONS, a computer program designed by B. May and C.C. Krueger and written in C by W. Eng.

Results

All populations of zebra mussels examined in this study had high levels of heterozygosity, varying between 0.27 and 0.43 (Table 2). The European populations had a slightly lower range of average heterozygosities (0.27–0.35) than the North American populations (0.30–0.43; $P < 0.05$ using the Mann–Whitney rank test). However, the range of Nei's genetic distance coefficients was higher among European populations than among North American populations (0.007–0.139 versus 0.005–0.025; Table 3, Fig. 3). Most of the differences between populations were due to differences in allelic frequencies at a number of loci, rather than the presence or absence of a rare allele at a single locus. The European populations (except Hungary) and the North American populations clustered separately with a distance between the clusters of 0.058.

F-statistics show that there was less differentiation among populations within North America than within Europe (F_{ST} = 0.019 vs. 0.07; Table 4). However, differentiation among populations within geographic units, i.e., each of the Great Lakes in North America and adjacent populations in Europe (Netherlands, Russia), was similar (0.07–0.021 vs. 0.015; Table 4). Significant differences were detected among all of the comparisons that we made, summed over all loci (Table 4).

Rare alleles were found at *DIA**, *GAPDH**, *GPI** (4 alleles), *MDH-1**, and *TPI** in North American populations that were not present in European populations, and at

Table 2. Allelic frequencies at 15 polymorphic loci in 25 populations of zebra mussels from North America and Europe. Allele

Locus/ allele	Alpe	Boll	Cape	Cats	Clair	Colch	Erie1	Erie2	Gary	Green	Grim	Holl	Mass	Owen
DIA														
0	0.03	—	0.03	—	—	—	0.03	0.01	0.01	0.02	—	0.01	—	—
100	0.69	0.67	0.69	0.63	0.63	0.50	0.62	0.68	0.55	0.68	0.69	0.71	0.67	0.58
150	0.18	0.05	0.13	0.21	0.21	0.23	0.14	0.21	0.22	0.15	0.16	0.14	0.16	0.19
160	0.01	0.03	0.03	0.01	0.01	0.05	0.02	0.01	0.03	0.03	—	0.01	0.01	0.02
190	0.01	0.07	—	0.04	0.04	0.05	0.05	0.01	0.05	0.03	—	0.01	0.01	0.03
200	0.08	0.18	0.12	0.10	0.12	0.18	0.14	0.08	0.14	0.09	0.14	0.12	0.15	0.19
N	39	38	73	35	43	42	74	39	39	76	34	76	69	72
EST														
100	0.68	0.73	0.65	0.80	0.66	0.63	0.70	0.61	0.75	0.73	0.66	0.69	0.70	0.69
105	0.33	0.27	0.36	0.20	0.34	0.37	0.30	0.39	0.25	0.27	0.34	0.31	0.30	0.32
N	40	39	76	40	44	42	80	40	26	75	35	80	71	65
FDH														
86	0.09	0.04	0.12	0.11	0.16	0.11	0.17	0.12	0.13	0.20	0.11	0.12	0.08	0.14
100	0.91	0.96	0.88	0.89	0.84	0.89	0.83	0.88	0.87	0.80	0.89	0.88	0.92	0.86
N	40	27	65	40	44	31	78	34	38	72	36	73	60	70
FH														
63	0.35	0.30	0.26	0.18	0.28	0.33	0.38	0.25	0.23	0.26	0.27	0.32	0.32	0.25
69	0.10	0.03	0.03	0.01	0.05	0.04	0.04	—	—	0.04	0.01	0.01	0.05	0.02
100	0.55	0.68	0.72	0.80	0.67	0.63	0.59	0.75	0.77	0.69	0.72	0.68	0.63	0.73
N	40	37	74	38	44	42	80	36	31	72	37	74	77	77
GAPDH														
97	—	—	—	0.03	0.03	0.01	—	—	—	—	—	—	—	—
100	0.73	0.45	0.58	0.53	0.56	0.74	0.86	0.69	0.65	0.53	0.54	0.72	0.52	0.69
123	0.28	0.55	0.42	0.45	0.41	0.25	0.14	0.31	0.36	0.47	0.46	0.28	0.48	0.31
N	40	39	74	40	45	42	80	35	31	77	39	79	76	74
GPI														
21	—	—	0.02	—	—	—	0.01	—	0.01	—	0.01	—	—	—
50	0.04	—	0.01	0.01	—	0.02	0.03	0.03	—	0.03	0.03	0.03	0.05	0.02
83	—	—	—	—	—	—	0.01	0.01	—	—	—	—	0.01	—
100	0.34	0.31	0.31	0.30	0.39	0.27	0.33	0.23	0.37	0.35	0.39	0.36	0.31	0.31
121	—	0.04	—	0.01	0.01	—	0.01	—	—	—	—	—	—	—
146	0.45	0.54	0.44	0.50	0.48	0.50	0.44	0.51	0.53	0.46	0.41	0.39	0.49	0.50
188	0.01	—	0.01	—	—	—	0.01	0.01	—	—	—	—	—	—
196	0.16	0.12	0.20	0.15	0.12	0.20	0.16	0.21	0.09	0.13	0.15	0.23	0.14	0.17
208	—	—	0.01	0.03	—	—	0.01	—	—	0.03	0.01	—	0.01	—
N	40	39	78	40	45	42	80	40	38	80	39	79	80	80
IDH														
91	0.33	0.27	0.31	0.27	0.27	0.35	0.32	0.32	0.42	0.39	0.28	0.24	0.29	0.34
100	0.68	0.73	0.69	0.73	0.73	0.66	0.68	0.68	0.58	0.61	0.73	0.76	0.70	0.67
114	—	—	—	—	—	—	—	—	—	—	—	—	0.01	—
89	—	—	—	—	—	—	—	—	—	—	—	—	—	—
N	40	39	72	39	44	42	80	39	37	79	40	80	80	79
MDH1														
77	—	—	0.01	—	—	—	0.01	—	—	0.01	—	—	—	0.01
100	0.60	0.63	0.56	0.55	0.50	0.52	0.61	0.64	0.62	0.58	0.61	0.65	0.60	0.53
120	0.40	0.38	0.43	0.45	0.50	0.48	0.39	0.36	0.38	0.41	0.39	0.35	0.40	0.47
N	40	40	77	40	45	42	80	40	38	79	32	80	78	78
MDH2														
100	0.86	0.91	0.91	0.95	0.87	0.80	0.87	0.85	0.86	0.89	0.94	0.89	0.85	0.94
140	0.14	0.10	0.09	0.05	0.13	0.20	0.13	0.15	0.14	0.11	0.06	0.11	0.15	0.06
180	—	—	—	—	—	—	—	—	—	—	—	—	—	—
N	40	37	74	40	45	42	80	40	39	80	40	80	78	78

mobilities are given in the left-hand column. Abbreviations for sampling locations are given in Table 1.

Palm	Roch	Sheb	Zion	Hung	Enkh	Peter	Roer	Volga	Vuren	Polan
—	—	—	—	—	—	—	—	0.02	—	—
0.73	0.66	0.63	0.68	0.99	0.97	0.98	0.97	0.82	0.91	0.85
0.15	0.19	0.24	0.18	—	—	—	—	0.03	—	0.03
0.03	—	0.02	—	—	—	—	—	—	—	—
0.03	0.01	0.04	0.04	—	—	—	—	0.02	—	—
0.06	0.14	0.08	0.10	0.01	0.03	0.02	0.03	0.12	0.10	0.12
39	35	72	74	40	37	29	32	30	37	78
0.63	0.89	0.60	0.72	0.64	0.89	0.91	0.69	0.74	0.75	0.76
0.37	0.11	0.40	0.28	0.36	0.11	0.09	0.30	0.26	0.25	0.24
39	14	79	79	28	35	29	29	29	36	76
0.14	0.08	0.10	0.16	0.65	0.11	0.11	0.18	0.09	0.07	0.18
0.86	0.92	0.90	0.84	0.35	0.89	0.89	0.82	0.91	0.93	0.82
39	26	77	70	13	35	22	37	28	35	76
0.39	0.29	0.28	0.25	0.36	0.25	0.07	0.15	0.13	0.27	0.32
—	0.01	0.03	0.05	—	—	—	—	—	—	0.02
0.61	0.70	0.69	0.70	0.65	0.75	0.93	0.85	0.87	0.73	0.66
36	35	77	77	31	38	27	39	30	37	80
—	—	—	0.01	—	—	—	—	—	—	—
0.63	0.61	0.70	0.61	0.75	0.36	0.36	0.24	0.27	0.30	0.30
0.37	0.39	0.30	0.38	0.25	0.64	0.64	0.76	0.73	0.70	0.70
39	35	68	78	38	39	28	39	26	38	76
0.01	—	—	—	—	—	—	—	—	—	—
0.04	0.01	0.02	0.01	—	0.01	—	—	0.05	0.01	—
—	—	—	—	—	—	—	—	—	—	—
0.30	0.31	0.35	0.30	0.14	0.33	0.38	0.40	0.27	0.20	0.39
—	—	0.05	—	—	—	—	—	—	—	—
0.47	0.44	0.48	0.51	0.73	0.45	0.33	0.42	0.54	0.58	0.51
—	—	—	0.01	—	—	—	0.01	—	—	0.01
0.18	0.21	0.11	0.17	0.14	0.21	0.28	0.17	0.14	0.21	0.09
—	0.03	—	0.01	—	—	—	—	—	—	—
39	40	78	77	40	38	30	28	28	40	80
0.23	0.29	0.50	0.30	0.36	0.69	0.67	0.67	0.50	0.67	0.54
0.77	0.70	0.50	0.69	0.64	0.31	0.33	0.33	0.47	0.33	0.46
—	0.01	—	0.01	—	—	—	—	0.02	—	—
—	—	—	—	—	—	—	—	0.02	—	—
39	38	74	69	40	39	29	39	29	39	75
—	0.01	—	0.01	—	—	—	—	—	—	—
0.54	0.63	0.59	0.59	0.47	0.68	0.70	0.81	0.82	0.81	0.55
0.46	0.36	0.41	0.41	0.53	0.32	0.30	0.19	0.18	0.19	0.45
39	40	80	80	39	39	30	39	30	39	80
0.89	0.81	0.89	0.92	0.80	0.90	0.85	0.94	0.84	0.95	0.89
0.12	0.19	0.11	0.08	0.20	0.10	0.12	0.06	0.11	0.05	0.11
—	—	—	—	—	—	0.03	—	0.05	—	—
39	37	78	78	40	39	30	39	28	37	80

Table 2 (*concluded*).

Locus/ allele	Alpe	Boll	Cape	Cats	Clair	Colch	Erie1	Erie2	Gary	Green	Grim	Holl	Mass	Owen
PAP														
100	0.65	0.75	0.69	0.53	0.54	0.58	0.67	0.59	0.59	0.66	0.59	0.67	0.69	0.69
114	0.35	0.25	0.31	0.47	0.46	0.42	0.33	0.41	0.41	0.34	0.41	0.33	0.31	0.31
N	39	34	74	37	41	26	79	38	34	74	39	72	74	75
PGD														
100	0.95	1.00	0.83	0.93	0.94	0.92	0.91	0.90	0.91	0.91	0.93	0.89	0.91	0.92
108	—	—	0.12	—	—	—	—	—	0.06	—	—	—	—	0.01
123	0.05	—	0.05	0.07	0.06	0.08	0.08	0.10	0.01	0.09	0.08	0.12	0.09	0.07
130	—	—	—	—	—	—	0.01	—	0.01	—	—	—	0.01	—
N	40	37	74	36	45	42	77	34	39	78	40	74	79	77
PGM1														
88	—	0.01	—	—	—	—	—	—	—	—	—	0.01	—	0.01
96	—	—	—	—	—	—	0.01	—	—	—	0.01	—	—	—
100	0.90	0.91	0.91	0.86	0.88	0.87	0.87	0.96	0.90	0.88	0.89	0.93	0.82	0.88
107	—	—	0.01	0.01	—	0.01	—	—	—	—	—	—	0.02	—
111	0.10	0.08	0.08	0.13	0.13	0.12	0.13	0.04	0.10	0.12	0.10	0.06	0.16	0.12
N	40	37	73	40	44	41	80	40	30	76	39	80	78	72
PGM2														
95	0.13	—	—	0.04	0.10	0.06	0.03	—	0.04	0.07	0.03	0.05	—	0.01
96	—	—	—	0.03	0.02	0.02	0.01	—	0.04	—	0.02	0.01	—	0.05
100	0.63	0.64	0.64	0.60	0.53	0.54	0.69	0.71	0.72	0.73	0.59	0.62	0.80	0.73
108	0.18	0.36	0.23	0.21	0.26	0.31	0.28	0.26	0.19	0.19	0.33	0.23	0.19	0.19
113	0.08	—	0.14	0.13	0.09	0.07	—	0.03	—	0.01	0.03	0.09	0.01	0.03
N	40	36	74	40	45	41	72	40	34	71	33	80	74	74
PP														
80	—	—	—	—	—	—	—	—	—	—	—	—	—	—
100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
N	40	40	78	40	45	43	80	40	39	80	40	80	80	80
TPI														
69	0.05	—	0.22	0.01	0.04	0.04	0.03	0.04	0.01	0.03	0.04	0.03	—	0.03
100	0.69	0.77	0.61	0.61	0.59	0.70	0.74	0.60	0.65	0.60	0.51	0.73	0.55	0.65
112	—	—	—	0.01	—	—	0.04	—	—	—	—	—	—	0.01
115	0.21	0.23	0.17	0.36	0.36	0.24	0.18	0.34	0.28	0.36	0.41	0.24	0.43	0.29
121	0.05	—	—	—	0.01	0.02	0.02	0.03	0.06	0.01	0.04	—	0.02	0.03
N	40	39	76	40	45	42	80	40	36	80	39	79	80	79
Mean HS	0.38	0.33	0.39	0.37	0.40	0.40	0.37	0.36	0.37	0.38	0.37	0.36	0.37	0.37
Mean SE	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.04	0.05	0.05	0.04	0.05
Mean H_o	0.39	0.31	0.35	0.37	0.40	0.43	0.37	0.35	0.36	0.37	0.36	0.33	0.33	0.35
SE	0.06	0.05	0.05	0.05	0.05	0.05	0.04	0.05	0.05	0.04	0.05	0.05	0.04	0.05

*IDH**, *MDH-2**, and *PP** we found rare alleles that were present in Europe but not in North America. We were unable to determine whether these alleles are the same as those detected by Hebert et al. (1989) and Boileau and Hebert (1993) as they did not record relative mobilities of allelic products in their studies. All of the rare alleles were present at such low frequencies that the likelihood of missing them if they were present in any one of the other populations was greater than 0.95 (Gregorius 1980). However, the probability that we missed all eight North American rare alleles if they were all present in at least one of the European populations is close to zero ($<10^{-7}$), i.e., the product of the probabilities of missing each allele separately in all samples.

Tests for deviations from Hardy–Weinberg expected genotype frequencies using $\alpha = 0.05$ revealed a high number of deviations (13%). Note that the frequency of such deviations can be reduced by using larger sample sizes. Using Bonferroni's procedure to correct for multiple tests made simultaneously, only two tests were significant: *PGD** in the population from IJsselmeer in the Netherlands and *DIA** in the population from Grimsby, Ont., on Lake Ontario (Snedecor and Cochran 1980; see also Cooper 1968). However, as Bonferroni's procedure is very conservative, it was worth looking for patterns in the tests that were significant at the 0.05 level. Using this criterion, eight deviations from Hardy–Weinberg expected genotype frequencies were found at *PGM-2** and nine were

Palm	Roch	Sheb	Zion	Hung	Enkh	Peter	Roer	Volga	Vuren	Polan
0.60	0.53	0.69	0.71	0.68	0.60	0.57	0.50	0.73	0.63	0.61
0.41	0.47	0.31	0.29	0.32	0.40	0.43	0.50	0.27	0.38	0.39
37	30	77	70	28	36	27	37	30	36	71
1.00	0.92	0.94	0.86	0.82	0.67	0.67	0.55	0.83	0.69	0.74
—	0.06	—	0.13	—	0.13	—	—	—	0.19	0.01
—	0.03	0.06	0.02	0.18	0.20	0.27	0.45	0.10	0.12	0.25
—	—	—	—	—	—	0.06	—	0.08	—	0.01
38	36	72	64	39	38	24	38	26	37	72
—	—	—	—	—	—	0.09	—	—	—	—
—	—	—	0.01	—	—	—	—	—	—	0.01
0.93	0.92	0.88	0.87	0.96	0.95	0.83	0.95	0.93	0.96	0.76
—	—	0.02	—	—	—	—	—	—	—	0.01
0.07	0.08	0.10	0.13	0.04	0.05	0.09	0.05	0.07	0.04	0.21
38	30	77	78	40	39	29	38	30	39	70
0.07	0.04	0.01	0.02	0.14	—	—	0.01	—	—	0.02
—	—	0.12	—	—	0.01	—	—	0.12	—	0.01
0.71	0.83	0.66	0.71	0.73	0.88	0.97	0.82	0.80	0.93	0.79
0.20	0.11	0.18	0.22	0.06	0.11	0.03	0.12	0.08	0.07	0.14
0.03	0.01	0.03	0.05	0.06	—	—	0.04	—	—	0.03
38	35	73	58	39	37	29	37	30	38	75
—	—	—	—	—	0.01	—	—	—	—	—
1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00
39	40	80	80	40	40	30	40	30	40	80
0.03	0.01	0.03	0.04	—	—	—	—	—	—	—
0.69	0.54	0.71	0.68	0.80	0.51	0.18	0.55	0.47	0.58	0.41
—	0.01	0.01	—	—	0.01	—	—	0.05	0.01	—
0.28	0.42	0.24	0.27	0.20	0.47	0.82	0.45	0.48	0.41	0.57
—	0.01	0.01	0.01	—	—	—	—	—	—	0.02
39	37	80	80	38	39	30	39	30	39	73
0.35	0.35	0.37	0.37	0.34	0.32	0.29	0.32	0.33	0.31	0.38
0.05	0.05	0.05	0.04	0.04	0.05	0.05	0.05	0.04	0.05	0.04
0.33	0.30	0.35	0.35	0.28	0.30	0.27	0.31	0.33	0.29	0.35
0.05	0.05	0.05	0.04	0.04	0.05	0.04	0.05	0.05	0.05	0.04

found at *DIA**. Given the high numbers of alleles found at many loci, including those that could not be resolved owing to crowding of the bands, it is likely that null alleles could have been present at these two loci, giving the appearance of deviations from expected Hardy–Weinberg genotype frequencies (heterozygote deficiencies). The majority (>80%) of the deviations detected at all loci at the 0.05 level were due to a deficiency of the common heterozygote. This is confirmed by the F_{IS} values, that are all positive (Table 4). Heterozygote deficiency has been frequently noted in bivalve molluscs, but no consensus explanation for this aberration has been proposed (Zouros and Foltz 1984; Mallet et al. 1985).

Discussion

Our data indicate that the zebra mussel populations within the Great Lakes and adjacent waters are not highly differentiated. The most genetically similar populations should be those from adjacent areas or areas linked by water flow. However, populations that were widely separated geographically, such as those from Massena, N.Y. and Green Bay, Wis. (D = 0.005), had genetic distances comparable with those from adjacent populations such as Gary, Ind., and Zion, Ill., in Lake Michigan (D = 0.006). The range of genetic distances among the North American populations (0.004–0.028) was smaller than has been detected in other mollusc species, for example, among 16 populations of two

Table 3. Nei's genetic distance coefficients calculated in 25 populations of zebra mussels using 15 polymorphic allozyme loci.

	Erie1	Erie2	Boll	Colch	Grim	Cape	Roch	Holl	Green	Zion	Sheb	Gary	Owen	Alpe	Palm
Clair	0.020	0.011	0.017	0.009	0.005	0.012	0.017	0.012	0.009	0.010	0.016	0.011	0.010	0.011	0.007
Erie1		0.013	0.024	0.009	0.022	0.018	0.024	0.007	0.018	0.011	0.012	0.014	0.009	0.006	0.012
Erie2			0.018	0.010	0.009	0.010	0.014	0.007	0.010	0.008	0.010	0.008	0.007	0.009	0.007
Boll				0.022	0.013	0.014	0.024	0.015	0.012	0.010	0.020	0.017	0.015	0.017	0.014
Colch					0.016	0.016	0.023	0.010	0.018	0.013	0.011	0.011	0.009	0.008	0.011
Grim						0.011	0.015	0.011	0.007	0.010	0.017	0.012	0.010	0.014	0.010
Cape							0.021	0.009	0.010	0.005	0.014	0.013	0.009	0.011	0.011
Roch								0.017	0.011	0.013	0.025	0.010	0.015	0.017	0.016
Holl									0.012	0.007	0.012	0.012	0.008	0.004	0.007
Green										0.005	0.011	0.007	0.007	0.011	0.010
Zion											0.010	0.007	0.004	0.008	0.008
Sheb												0.008	0.007	0.008	0.014
Gary													0.006	0.011	0.013
Owen														0.008	0.009
Alpe															0.006
Palm															
Cats															
Mass															
Polan															
Hung															
Vuren															
Enkh															
Roer															
Peter															
Volga															

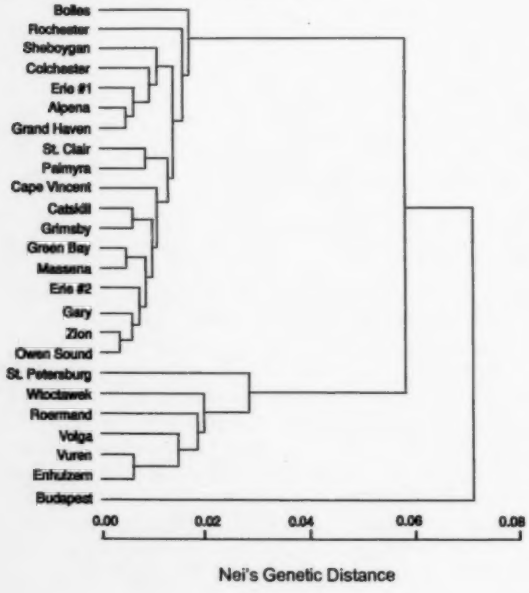


Fig. 3. Relative genetic distance dendrogram generated by UPGMA analysis of Nei's (1972) genetic distance coefficients calculated in 25 populations of zebra mussels using 15 polymorphic allozyme loci.

species of North American unionid bivalves (0.023–0.184; Davis 1983). Therefore, in the 6 years since the zebra mussels entered North America, they have not undergone any genetic differentiation, microevolution, or natural selection for traits linked to protein-coding loci. This result suggests that, as the mussels expand their range, any differentiation due to small founder populations has been overwhelmed by frequent genetic mixing with new immigrants. This result is not surprising. The mussels spread throughout the Great Lakes in 4 years, and throughout the major river drainages within 6 years of their introduction. This rate of spread suggests that mussels are translocated very easily, particularly downstream of established populations owing to the passive movement of veligers. Consequently, the likelihood of reintroduction to many of these locations on a regular basis is quite high. The size of many zebra mussel vectors (ballast tanks, intra- and inter-lake currents) suggests that each new introduction probably comprises a large number of animals. Thus, founder effect is unlikely to occur, and continual genetic mixing of established populations ensures homogeneity among populations. The effects of natural selection, which would have to be quite strong to have occurred within 6 years (a hypothetical maximum of 12 zebra mussel generations), would also be lost owing to such mixing. This being the case, the only areas where we would be likely to find genetically differentiated populations would be in isolated bodies of water that have low probabilities for repetitive

These numbers were used to generate the dendrogram in Fig. 3.

Cats	Mass	Polan	Hung	Vuren	Enkh	Roer	Peter	Volga
0.006	0.012	0.038	0.061	0.063	0.058	0.074	0.090	0.050
0.026	0.021	0.067	0.050	0.075	0.073	0.099	0.118	0.071
0.012	0.011	0.044	0.054	0.047	0.049	0.063	0.079	0.039
0.016	0.012	0.043	0.075	0.048	0.053	0.073	0.097	0.035
0.017	0.020	0.062	0.062	0.076	0.075	0.097	0.119	0.069
0.007	0.008	0.031	0.073	0.048	0.047	0.058	0.071	0.035
0.014	0.014	0.043	0.059	0.048	0.050	0.066	0.088	0.041
0.011	0.010	0.036	0.074	0.043	0.037	0.065	0.058	0.036
0.014	0.014	0.054	0.054	0.059	0.058	0.075	0.097	0.051
0.009	0.005	0.021	0.050	0.033	0.030	0.047	0.059	0.025
0.010	0.008	0.038	0.050	0.043	0.044	0.066	0.082	0.037
0.020	0.016	0.046	0.059	0.049	0.050	0.069	0.090	0.044
0.010	0.012	0.039	0.061	0.043	0.042	0.063	0.075	0.036
0.010	0.009	0.042	0.055	0.053	0.053	0.075	0.086	0.044
0.017	0.013	0.052	0.054	0.060	0.059	0.083	0.102	0.055
0.013	0.010	0.046	0.048	0.058	0.059	0.080	0.099	0.051
	0.012	0.035	0.070	0.051	0.047	0.063	0.073	0.039
		0.023	0.067	0.039	0.039	0.059	0.064	0.028
			0.083	0.022	0.015	0.024	0.027	0.021
				0.094	0.090	0.106	0.139	0.098
					0.007	0.017	0.034	0.013
						0.015	0.018	0.018
							0.028	0.024
								0.031

reinfestation. If any of these isolated populations are found to have quite different characteristics from the Great Lakes populations, investigators would be advised to examine the genetic structure of the population before concluding that such differences are due only to environmental factors. Relatively isolated populations are likely to differentiate from source populations over time. Thus, the data presented here serve as baseline data to which similar studies can be compared in several years to determine whether genetic divergence of populations has taken place.

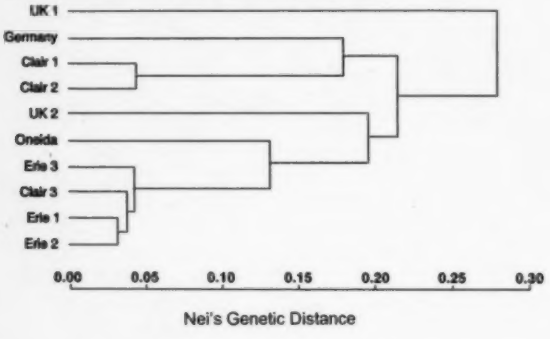
The closest genetic relationships among populations we examined were between the two populations from Lake Huron and between the two from the Netherlands. Lake Huron may have only been invaded once, as the lake is upstream of all other populations except the tenuous colony in Duluth Harbor, and there are few major ports on Lake Huron relative to the other lakes. The Netherlands populations are geographically close, but one would expect this busy shipping area to receive multiple introductions from different locations in Europe.

Boileau and Hebert (1993) observed much higher allelic frequency differences among populations than found in this study. A dendrogram that we constructed from Boileau and Hebert's data shows large genetic differences even between geographically adjacent populations (Fig. 4). For example, populations from within Lake St. Clair are separated by a genetic distance of 0.215, which is considerably greater than the maximum distance we observed between any populations, even between continents. These differences can only partly be explained by the small sample sizes used by Boileau and Hebert ($N = 24$ in most cases), which will increase the variance on the allelic frequency estimates and increase the probability of missing alleles present in low frequencies. Similar numbers of loci were used in both studies (15 in our study, 11 in theirs), although we found more alleles, on average, at each locus (4.2 vs. 3.3). However, none of these factors explains the large differences between populations detected in Boileau and Hebert's study, such as the frequency of *LG3*1* found in the three populations of Lake St. Clair (0.04, 0.27, and 0.65, respectively). Most of the mussels in both studies were collected during the same time period (summer 1990 through fall 1991), so differences are not likely to reflect new introductions or temporal changes in the populations. The extreme genetic distances noted by Boileau and Hebert, which are larger than species differences found in some taxonomic studies, could be explained in part if some of their populations were inadvertently comprised of a mixture of zebra and quagga mussels (*Dreissena bugensis*; Spidle et al. 1994). We unfortunately cannot resolve this discrepancy because Boileau and Hebert did not record allele mobilities nor preserve a record of their gels (M.G. Boileau, Department of Biology, University of Guelph, Guelph, ON N1G 2W1, personal communication).

Table 4. *F*- and *G*-test statistics calculated over 15 polymorphic allozyme loci observed in 25 populations of zebra mussels. *N* is the number of groups compared in a test.

	N	*G*	df	*p*	F_{IS}	F_{IT}	F_{ST}
Lakes St. Clair and Erie	5	299.191	160	$<1 \times 10^{-9}$	0.000	0.020	0.021
Lake Ontario	3	165.61	74	$<1 \times 10^{-8}$	0.100	0.115	0.018
Lake Michigan	5	365.058	160	$<1 \times 10^{-17}$	0.060	0.072	0.012
Lake Huron	2	65.275	36	0.002	0.001	0.006	0.007
Lakes Ontario, Erie, Huron, and Michigan	4	260.737	123	$<1 \times 10^{-11}$	0.054	0.058	0.004
North American riverine populations	3	141.349	74	$<1 \times 10^{-5}$	0.060	0.073	0.014
Riverine and Great Lakes populations	2	97.787	41	$<1 \times 10^{-5}$	0.072	0.074	0.003
All North American populations	18	1395.01	697	$<1 \times 10^{-48}$	0.043	0.061	0.019
Netherlands	2	55.054	28	0.0017	0.050	0.065	0.015
Russia	2	83.656	35	$<1 \times 10^{-5}$	0.030	0.062	0.015
Eastern Europe	3	325.884	64	$<1 \times 10^{-36}$	0.088	0.159	0.077
Eastern Europe, Netherlands, and Russia	3	263.108	80	$<1 \times 10^{-20}$	0.089	0.106	0.018
All European populations	7	727.661	240	$<1 \times 10^{-49}$	0.062	0.128	0.070
North American and European populations	2	931.997	44	$<1 \times 10^{-166}$	0.091	0.115	0.027

Fig. 4. Relative genetic distance dendrogram generated by UPGMA analysis of Nei's (1972) genetic distance coefficients calculated using data from Boileau and Hebert (1993) for 11 polymorphic allozyme loci. Abbreviations refer to sites described in Boileau and Hebert (1993): ER, Lake Erie; ON, Oneida Lake; SC, Lake St. Clair; UK, United Kingdom.



Genetic distances among the European populations examined in this study, with the exception of the population from Hungary, were similar to distances among the North American populations. Thus, either the European populations are still exchanging individuals with sufficient regularity to prevent divergence of allelic frequencies, or these populations have not been separated for sufficient time for genetic drift and consequent divergence to occur. The relatively minor differences in the average heterozygosity between European and North American populations suggest that little variation was lost when zebra mussels invaded North America, and therefore, we can conclude that the founder population comprised a relatively large number of individuals. We cannot eliminate the possibility that one or more of the populations we examined from Europe contributed to the populations in North America. However, the presence of eight unique alleles in North America and three in Europe, coupled with the difference observed between the North American and European population groupings, suggests that at least one major contributor to the invader population has yet to be found.

Detection of a rare allele that is present in North America but absent in a European population cannot be used to eliminate the European population as a potential invader, as inferred by Boileau and Hebert (1993), owing to the large probability of missing the allele with the sample sizes used in both our and their study (see Gregorius 1980). Although we found higher genetic distances between North America and Europe than among populations from these two areas, and we also found allelic frequency differences among populations from the two continents, these differences are not sufficiently large to eliminate the possibility of relatedness. The extremely high levels of genetic variability in zebra mussels, also noted by Hebert et al. (1989), Boileau and Hebert (1993), and others, are not unusual for molluscs, which tend to be highly variable at allozyme loci (e.g., Nevo 1983). However, these levels of heterozygosity require that large sample sizes be used for adequate estimation of allelic frequencies, average heterozygosity, and genetic distances when only a few loci are examined (i.e., less than 30; e.g., Nei 1978).

We may never be able to absolutely identify the founder populations from Europe, even with genetic data, if more than one population contributed to the invasion. However, a complete survey of possible founder populations may permit estimation of the relative probabilities that each population could be a founder. A logical place to look for possible founder populations is the Dnieper and Bug drainages of the Black Sea, which is the source of the quagga mussel (Spidle et al. 1994).

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Concentrations of nucleic acids and protein as indices of nutritional status for recently emerged brook trout (*Salvelinus fontinalis*)

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Abstract: We examined whether concentrations of nucleic acids and protein sampled from muscle tissue were useful indicators of nutritional status for recently emerged brook trout (*Salvelinus fontinalis*). Trout collected from the field were maintained in the laboratory under two food treatments: fed versus food deprived. Concentrations of RNA and DNA, and RNA/DNA ratio did not differ significantly between fed and food-deprived trout, but fed trout had higher concentrations of protein and were 20% heavier at a given fork length than food-deprived trout. Wild trout fed in the laboratory had lower concentrations of RNA, DNA, and protein, and lower RNA/DNA ratios than did trout in the field, but were significantly heavier for their fork length. Tissue concentrations of protein may provide an index of nutritional status for recently emerged brook trout while concentrations of nucleic acids apparently do not. It is important to ensure that concentrations of nucleic acids and protein reflect nutritional status adequately for the species and life stage of interest before using these indices to assess the condition of fish in the field.

Résumé : Nous avons cherché à savoir si les concentrations d'acides nucléiques et de protéines prélevées de tissus musculaires étaient de bons indicateurs de l'état nutritionnel d'ombles de fontaine (*Salvelinus fontinalis*) à l'état d'alevins nageant depuis peu. Des ombles recueillies dans la nature ont été conservées en laboratoire selon deux programmes : un groupe a été nourri, l'autre pas. Les concentrations d'ARN et d'ADN et le rapport ARN/ADN ne différaient pas significativement entre les deux groupes; toutefois, les ombles nourries présentaient des concentrations plus élevées de protéines et un poids supérieur de 20 %, à une longueur donnée à la fourche, que les ombles de l'autre groupe. Les poissons sauvages nourris en laboratoire présentaient des concentrations moins élevées d'ARN, d'ADN et de protéines ainsi que des rapports ARN/ADN moins élevés que les truites du milieu naturel, mais accusaient un poids significativement plus important, à longueur à la fourche égale. Les concentrations de protéines dans les tissus peuvent être un indicateur de l'état nutritionnel, dans le cas des alevins nageant depuis peu, ce qui n'est apparemment pas le cas des concentrations d'acides nucléiques. Il importe de s'assurer que les concentrations d'acides nucléiques et de protéines traduisent fidèlement l'état nutritionnel pour l'espèce et le stade de développement visé, avant d'utiliser ces indices pour évaluer l'état du poisson dans la nature.

[Traduit par la Rédaction]

Introduction

Biochemical indices such as tissue concentrations of RNA and the ratio of RNA/DNA are being used to assess the nutritional status and recent growth rates of fish. Protein synthesis is required for the production of new tissue. The concentration of RNA, which is predominantly ribosomal RNA, provides an index of the number of ribosomes per milligram of tissue, while the concentration of DNA estimates the number of cells per milligram of tissue. The ratio of RNA/DNA, therefore, provides an index of protein-synthesizing potential per cell. An impressive number of studies has demonstrated that fish fed on high rations tend to have higher concentrations of RNA and higher RNA/DNA ratios than fish deprived of food or maintained on low rations (Bulow 1987; Clemmensen 1987; Raae et al. 1988; Richard et al. 1991; Steinhart and Eckmann 1992). Some investigations have also demonstrated that these biochemical measures are positively correlated with short-term growth rates (Wilder and Stanley 1983; Miglavs and

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Jobling 1989; Ferguson and Danzmann 1990; Bastrop et al. 1992). In addition, several studies have generated equations using water temperature and RNA/DNA ratio to predict either the specific growth rates of fish sampled from the field or the length of time the fish have gone without food (Buckley et al. 1984; Clemmensen 1987; Mathers et al. 1992).

Two other indices have commonly been used to assess a fish's state of well-being. One is weight-at-length or relative condition factor (LeCren 1951), which is expected to be higher for fish that are well fed than it is for those that are poorly fed. Another is the tissue concentration of protein, which also tends to be higher for fed fish than for fish deprived of food (Smith 1981; Clemmensen 1987). Unfortunately, to date there has been little effort to compare these indices with those based on concentrations of nucleic acids.

We wanted to know whether variation in the behaviour exhibited by recently emerged brook trout (*Salvelinus fontinalis*) in the field was related to their nutritional status and, therefore, needed to identify a reliable index of nutritional status. At our study sites, the trout exhibit conspicuous variation in their use of still versus running water, their activity while foraging, and their aggressiveness (Grant and Noakes 1988; McLaughlin et al. 1992, 1994; McLaughlin and Grant 1994). In stream salmonids, such variation in foraging and social behaviour is believed to affect an individual's foraging success and survival and to be an important part of the mechanism determining cohort size (Latta 1969; Elliott 1989, 1990*a*, 1990*b*).

In a review of biochemical indices of growth in fish, Bulow (1987) emphasized that the indices be applied and interpreted with caution because they vary among species and life stages within a species, as well as across rearing environments. Much of the literature examining the biochemical indices in young fish has focused on marine fishes (e.g., Clemmensen 1987; Mathers et al. 1992; but see Mathers et al. 1993). Because no previous study has examined such indices in recently emerged brook trout, it was necessary for us to assess whether the indices provided useful indicators of nutritional status for the early life stages of this species. In our study, recently emerged trout were collected from the field, maintained in the laboratory for 7 d, and then exposed to one of two feeding regimes: fed for 8 d versus deprived of food for 8 d. We expected fed fish to have higher concentrations of RNA and protein, and higher RNA/DNA ratios, than fish deprived of food. Because we were interested in using the biochemical indices to assess the status of fish in the field, we compared measurements for the fed, wild trout held in the laboratory with measurements made for trout of similar size sampled directly from the field.

Materials and methods

Fed versus food-deprived trout in the laboratory

Twenty to 24 recently emerged brook trout (fork length: 20–35 mm) were captured using aquarium dip nets at each of three locations in Ontario, Canada: the West Branch of the Credit River 2 km northeast of Erin (43°45′N, 80°07′W); the North Branch of the Credit River upstream of Highway 24 near Caledon (43°52′N, 80°00′W); and Black Creek near Limehouse (43°38′N, 79°59′W). The sites were sampled on 6, 7, and 28 April 1992, respectively. Studies of the behaviour and ecology of brook trout have been carried out at these sites since 1984. These drainages were stocked until the early 1970s (Ontario Ministry of Natural Resources, personal communication), but recent studies of mitochondrial DNA provided no evidence of hatchery stock remaining at these sites (R. Danzmann, unpublished data).

In the laboratory, the trout were kept in well water held at 8–9°C. The water circulation system was closed, and 10–20% of the water was removed and replaced with fresh well water each day. Photoperiod corresponded with the natural photoperiod at this time of year, approximately 13 h light : 11 h dark.

The sample of fish from each site was maintained in an opaque Plexiglas container (49 × 39 × 31 cm) placed in a plastic wash basin. Each container was divided along its length with an opaque piece of Plexiglas to create two compartments each containing 10–12 trout. Each compartment had a false bottom made of fine fibreglass mesh and contained an aquarium filter. Rates of water flow into the compartments ranged from 29 to 35 $mL \cdot s^{-1}$.

For the 7 d following their arrival in the laboratory, the trout were hand fed BioDiet© three times daily at a ration three times that recommended by Bardach et al. (1972) for trout of this size. This allowed the trout to become accustomed to hatchery food and fish were observed eating within a couple of days after arrival in the laboratory. At the end of 7 d, one compartment in each container was selected at random and the fish deprived of food for 8 d (deprived treatment) while fish in the adjacent compartment continued to be fed as before (fed treatment). After 8 d, the trout were killed by a blow to the head, measured for fork length (mm), placed singly in plastic tubes, and frozen at −70°C. Recently emerged brook trout may withstand several weeks or more of food deprivation before dying (Latta 1969); however, 8 d of food deprivation was considered a reasonable length of time for our purpose of attempting to relate behaviour to nutritional status in the field.

Fed trout in the laboratory versus trout in the field

Forty-two recently emerged brook trout were sampled from our field sites between 4 and 26 April 1991 and another forty two between 4 April and 8 May 1992. These trout were sampled from still-water pools along the stream edge where current speeds were comparable with those in our laboratory containers. Each fish was killed, measured for fork length (mm), put in a plastic tube, and placed on wet ice. On return to the laboratory, the fish were frozen at −70°C. Ferguson and Drahushchak (1989) found that estimates of RNA and DNA concentrations were unaffected when samples were placed on wet ice for 24 h or less, rather than being frozen immediately. Means of the water temperature measured at the location where each specimen was sampled in the field were 10 ± 0.4°C (mean ± SE) and 9 ± 0.4°C for 1991 and 1992, respectively.

Table 1. Percentage of the total variance in indices of nutritional status accounted for by each source of variation based on two-way analysis of variance. Statistically significant ($P < 0.05$) sources of variation appear in bold.

	Index of nutritional status				
Source of variation	Weight at length	RNA concentration	RNA/DNA ratio	DNA concentration	Protein concentration
Sample site	0.0	**10.0**	**9.0**	0.0	**12.7**
Food treatment	**30.5**	0.0	0.0	0.0	**19.6**
Interaction	**7.5**	0.0	0.0	0.0	0.0
Error	62.0	90.0	91.0	100.0	67.7
No. of individuals	65	66	66	66	67

Nucleic acid and protein assays

Wet weights of the fish were measured prior to assaying for RNA, DNA, and protein. Following this, each specimen was decapitated and gutted. DNA and RNA concentrations were measured for the remaining, predominantly muscle tissue according to the fluorometric technique of Karsten and Wollenberger (1972, 1977), as described in Ferguson and Drahushchak (1989). Concentrations of protein were quantified using the technique of Bradford (1976) using gamma globulin as a standard. Estimates of protein concentrations were not made for trout sampled from the field in 1991.

Statistical analyses

Residuals calculated from regressions of $\log_{10}$(wet weight) versus $\log_{10}$(fork length) were used as our index of relative condition factor. Positive residuals indicate that fish are heavier on average for their length while negative residuals indicate that fish are lighter on average for their lenght.

For our laboratory experiment, proportions of the total variance due to differences among samples (West Credit, North Credit, or Black Creek) and food treatments (fed or deprived) were estimated for each of our condition variables using a two-way analysis of variance (ANOVA). Both sample and food treatment were treated as fixed effects. The magnitude of each fixed effect was estimated using the method described by Winer et al. (1991, p. 405).

For each index of nutritional status, trout sampled from the field in 1991 and 1992 were compared with those in the fed treatment of the laboratory experiment using a one-way ANOVA, with the exception of protein concentration where *t*-tests were used. Within each year, data from the different sample sites were combined for this comparison.

With our ANOVA, planned comparisons were tested using linear contrasts. Unplanned comparisons among specific laboratory and field samples were made using the method of Scheffé which maintains the experimentwise error rate at 0.05 (Day and Quinn 1989).

Results

Fed versus food-deprived trout in the laboratory

On average, trout in the fed treatment were heavier for their length than those in the deprived treatment ($F = 37.1$, $P < 0.0001$, df = 1, 59; Fig. 1*a*). Geometric mean fork length for all fish at the end of the experiment was 25 mm (minimum = 20 mm, maximum = 37 mm). After statistically adjusting for the variation in fork length, the overall mean wet weight for fed fish was 20% heavier than that for food-deprived fish (least-square means at 25 mm were 114 mg and 95 mg, respectively, $F = 35.5$, $P < 0.0001$, df = 1, 62). The fed fish were also more active and brighter in colour at the end of the experiment than were the food-deprived fish. Overall, 30.5% of the total variance in relative condition factor was attributed to direct differences between food treatments (Table 1).

RNA concentrations and RNA/DNA ratios did not differ significantly among the fed and food-deprived fish ($F = 0.5$, $P > 0.50$, df = 1, 60 and $F = 0.1$, $P > 0.70$, df =1, 60, respectively; Figs. 1*b* and 1*c*). Moreover, differences between food treatments did not account for any of the overall variance in either RNA concentration or RNA/DNA ratio (Table 1). DNA concentration also did not differ significantly among the fed and food-deprived fish ($F = 0.6$, $P > 0.40$, df = 1, 60; Table 1).

Protein concentrations were significantly higher for fed fish than for food-deprived fish ($F = 19.5$, $P < 0.0001$, df = 1, 61; Fig. 2). Differences among food treatments within samples accounted for 19.6% of the overall variance in protein concentration (Table 1). The difference between fed and food-deprived fish was 8.5 mg protein/g tissue, on average (95% confidence limits: 4.7–12.3).

Fed trout in the laboratory versus trout in the field

Trout from the fed treatment of the laboratory experiment were significantly heavier for their length than trout sampled directly from the field (*P*s < 0.05; Fig. 3*a*). At the geometric mean fork length of 24 mm, the least square estimates of wet weight were 102, 91, and 89 mg for the laboratory fish and for the field samples from 1991 and 1992, respectively. There was no significant difference among trout sampled from the field in 1991 versus 1992 ($P > 0.20$).

Trout in the laboratory had significantly lower concentrations of RNA and DNA, and lower RNA/DNA ratios than trout sampled from the field (*P*s < 0.05; Figs. 3*b*–3*d*). The same was true for protein ($t = -8.80$, $P < 0.001$,

Fig. 1. Box plots summarizing (*a*) relative condition factor, (*b*) RNA concentration, (*c*) RNA/DNA ratio, and (*d*) DNA concentration for fed (F) and food-deprived (D) brook trout in the laboratory. Each box plot indicates the 10th, 25th, 50th, 75th, and 90th percentiles by horizontal lines. All values below the 10th percentile and above the 90th percentile are indicated by individual data points.

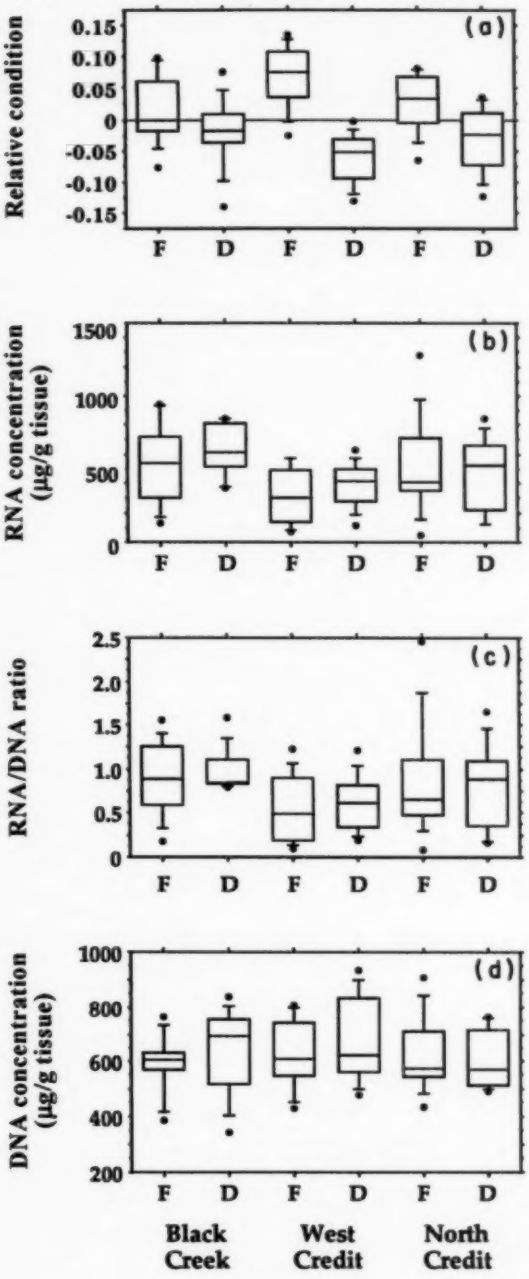
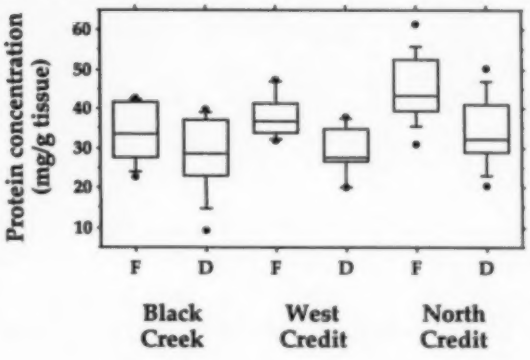


Fig. 2. Box plots summarizing the protein concentrations measured for fed (F) and food-deprived (D) brook trout in the laboratory.



df = 74; Fig. 4). No differences in RNA concentration, RNA/DNA ratio, and DNA concentration were detected between trout sampled from the field in 1991 versus those sampled in 1992 (all *P*s > 0.05).

Discussion

The tissue concentration of RNA and the ratio of RNA/DNA are apparently not suitable biochemical indices of nutritional status for recently emerged brook trout. These indices did not differ significantly despite the large differences in relative condition observed between fed and food-deprived fish in our laboratory experiment. In contrast, the concentration of protein may provide a potentially useful biochemical index of nutritional status for recently emerged brook trout, being significantly higher for fed fish than for food-deprived fish.

The lack of differences in RNA and RNA/DNA ratio between fed and food-deprived brook trout contrasts with the findings of numerous earlier studies where fed fish typically had higher concentrations of RNA and higher RNA/DNA ratios than food-deprived fish (see Introduction). Nevertheless, there have been other instances where nucleic acids were not found to be good indicators of nutritional status. In a study of young rainbow trout (*Oncorhynchus mykiss*), Mathers et al. (1993) reported little variation in RNA concentration and RNA/DNA ratio among fed and food-deprived trout at 21–30 d posthatching, the time when exogenous feeding had begun but some yolk reserves also remained. Yet considerable variation in growth rates (percent protein per day) was observed among the trout. Pronounced differences in RNA and RNA/DNA ratio were observed among the fed and food-deprived trout at 58–65 d posthatching, by which time the fish were relying solely on exogenous energy sources. Steinhart and Eckmann (1992) also reported that the difference in RNA/DNA ratio among fed and unfed whitefish (*Coregonus* spp.) larvae was greater when the larvae were older (>26 d posthatch) than when they were younger (13 d posthatch). In addition, several studies of marine fishes have reported high variability in RNA/DNA ratios during early life stages, and this variability

Fig. 3. Box plots summarizing (*a*) relative condition factor, (*b*) RNA concentration, (*c*) RNA/DNA ratio, and (*d*) DNA concentration for fed brook trout used in our laboratory experiment (L92) versus trout at the same stage of development sampled directly from the field during 1991 (F91) and 1992 (F92).

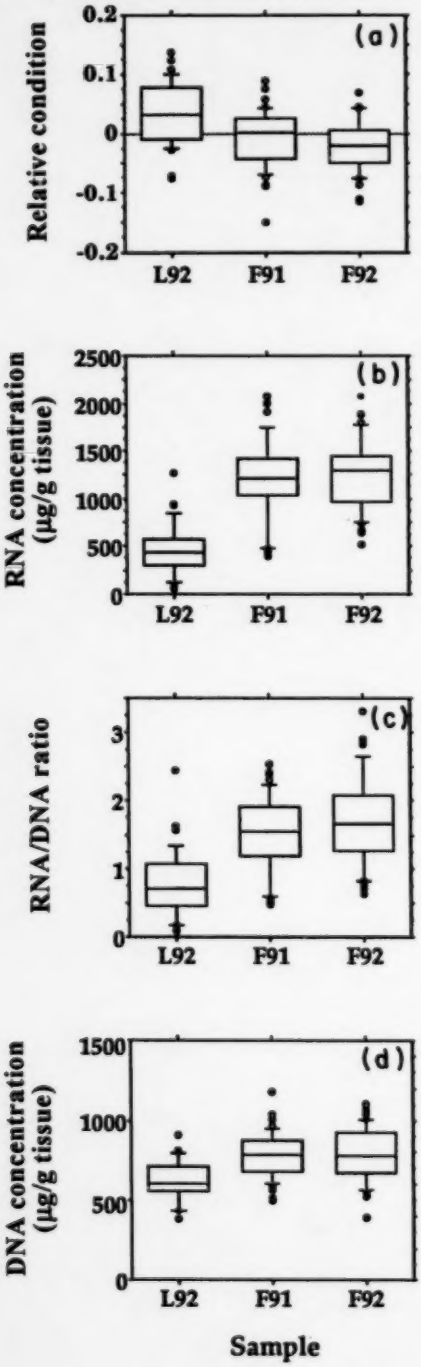
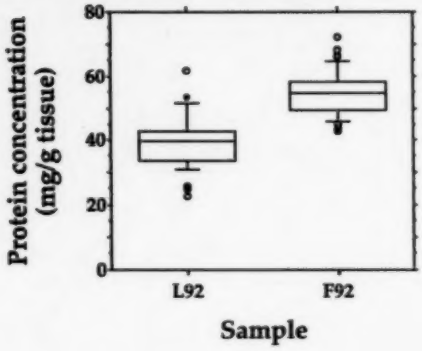


Fig. 4. Box plots summarizing protein concentration for the fed (F) brook trout used in our laboratory experiment (L92) versus trout at the same stage of development sampled directly from the field during 1992 (F92). Protein concentration was not measured in 1991.



impeded attempts to use RNA/DNA ratios to distinguish between fed and food-deprived fish (Westerman and Holt 1988; Richard et al. 1991).

Using nucleic acids to assess recent feeding history or growth rate assumes that the tissue concentration of RNA is influenced by the fish's recent feeding success while the activity of the ribosomes and the retention of synthesized protein as tissue remain relatively constant. This assumption can be incorrect (Smith 1981; Loughna and Goldspink 1984). Therefore, variation in the rate of translation or protein retention, rather than the amount of RNA, could explain our findings, and changes in these processes have been used in the past to explain apparently anomalous results. Miglavs and Jobling (1989), for example, found that juvenile arctic char (*Salvelinus alpinus*) deprived of food and then subsequently refed exhibited faster (compensatory) growth than char fed continuously, even though the former group had lower RNA/DNA ratios than the latter. Mathers et al. (1993) postulated that rainbow trout did not adapt their RNA concentrations to feeding success during the first 30 d posthatching because the fish had all the RNA required for protein synthesis and protein growth to proceed. The variation in growth rate was attributed to variation in RNA activity. Indeed, with rainbow trout the initial response to food deprivation is a reduction in RNA activity followed later by a reduction in the concentration of RNA (Loughna and Goldspink 1984). It is possible, therefore, that we might have detected differences in RNA concentration and RNA/DNA ratio between fed and food-deprived brook trout had the experiment been conducted for a period greater than 8 d.

With stream-dwelling salmonids, maintaining RNA concentrations during short periods of food deprivation may be important immediately after emergence. The transition from endogenous to exogenous energy sources is considered to be a critical period in an individual's development (Kamler 1992) and, in the field, high mortality is often observed at this time (Elliott 1989; Grant and Kramer 1990). Perhaps during or shortly after this transition

food-deprived trout rely on endogenous sources of energy, such as triacylglycerol (Fraser 1989), to prevent RNA concentrations from declining, possibly because any loss of protein synthesizing machinery would impede a starving individual's chances of recovery should feeding conditions improve. Recently emerged brook trout can withstand 1–2 months of food deprivation before dying, but fish deprived of food for several weeks and then refed may exhibit high mortality after food has been made available (Latta 1969).

In our experiments, the brook trout provided with food had higher tissue concentrations of protein than trout that were not provided with food suggesting that, in recently emerged trout, the tissue concentration of protein is a better indicator of nutritional status than the concentration of RNA or the ratio of RNA to DNA. Several earlier studies of salmonids in particular, and fishes in general, have reported higher tissue concentrations of protein in fed versus food-deprived fish (Parker and Vanstone 1966; Smith 1981; Clemmensen 1987; Richard et al. 1991). Nevertheless, protein concentration has received far less attention than RNA/DNA ratio in recent investigations of biochemical indices of growth and nutritional status. Clemmensen (1987) considered protein concentration to be less reliable than RNA/DNA ratio for assessing the nutritional status of herring (*Clupea harengus*) and turbot (*Scophthalmus maximus*) larvae although the basis for this conclusion was unclear. Moreover, many studies examining RNA/DNA ratio as an index of nutritional status have not considered protein concentration as an alternative. Future comparisons of condition indices might also consider triacylglycerol, a storage lipid used when young fish cannot attain their energy requirements exogenously (Fraser 1989).

The biochemical indices are probably best considered to be relative rather than absolute indicators of nutritional status. Concentrations of RNA, DNA, and protein, as well as ratios of RNA/DNA were all higher for the trout we sampled directly from the field than they were for the trout we maintained in the laboratory, even though the laboratory trout were heavier for a given length. Several earlier studies have reported higher RNA/DNA ratios for fish growing under natural conditions versus those reared in the laboratory (Wilder and Stanley 1983; Buckley 1984; Richard et al. 1991). The reasons for such differences are unknown, but such findings suggest that these biochemical indices can be influenced by factors other than the amount of food consumed, such as handling, changes in food quality or water chemistry, or differences in rearing substrates or densities (e.g., Steinhart and Eckmann 1992; Sveier and Raae 1992).

Our inability to detect differences in RNA/DNA ratio between fed and food-deprived fish needs to be considered in light of two additional issues. One issue is the number of very different methods available for measuring concentrations of RNA and DNA, and the lack of any standard protocol (McGurk and Kusser 1992; Grémare and Vétion 1994). Our protocol was a modification of the Karsten and Wollenberger method (1972, 1977). In a study of herring larvae (*Clupea pallasi*), McGurk and Kusser (1992) concluded that the Karsten and Wollenberger method was less reliable than methods that include steps to purify the nucleic acids. However, other studies applying the Karsten and Wollenberger method have detected differences in RNA/DNA ratio between fed and food-deprived fish (Richard et al. 1991; R.L. McLaughlin, unpublished data), or detected positive correlations between RNA concentration and recent growth rate in salmonid fishes (Ferguson and Danzmann 1990; Arndt et al. 1995). In addition, Grémare and Vétion (1994) have recently advised against using steps to purify the nucleic acids.

A second issue concerns the higher concentrations of RNA, DNA, and protein we found for brook trout sampled in the field relative to the fed fish in our experiment. This raises the possibility that fed fish in the laboratory experiment were exhibiting little growth while the starved fish were metabolizing protein. We consider this unlikely because other samples of recently emerged trout used for genetic studies were collected from our study sites during the same period and housed under the same conditions as the trout used in our feeding experiment. The former grew successfully and several individuals were sexually mature 10 months later.

Our study underscores the importance of verifying for the species and the life stage of interest whether the various biochemical indices adequately reflect a fish's nutritional status. While the rationale behind RNA/DNA ratios appears simple and is intuitively appealing, there may be circumstances where the concentration of RNA or the ratio of RNA/DNA are not good indices of nutritional status or recent growth rate. In such circumstances, the tissue concentration of protein may provide a suitable alternative.

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Estimating the influence of temperature on the survival of chinook salmon smolts (*Oncorhynchus tshawytscha*) migrating through the Sacramento – San Joaquin River Delta of California

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Abstract: Data from the U.S. Fish and Wildlife Service are used to investigate the relationship between water temperature and survival of hatchery-raised fall-run chinook salmon (*Oncorhynchus tshawytscha*) smolts migrating through the Sacramento – San Joaquin Delta of California. A formal statistical model is presented for the release of smolts marked with coded-wire tags (CWTs) in the lower Sacramento River and the subsequent recovery of marked smolts in midwater trawls in the Delta. This model treats survival as a logistic function of water temperature, and the release and recovery of different CWT groups as independent mark–recapture experiments. Iteratively reweighted least squares is used to fit the model to the data, and simulation is used to establish confidence intervals for the fitted parameters. A 95% confidence interval for the upper incipient lethal temperature, inferred from the trawl data by this method, is 23.01 ± 1.08°C This is in good agreement with published experimental results obtained under controlled conditions (24.3 ± 0.1 and 25.1 ± 0.1°C for chinook salmon acclimatized to 10 and 20°C, respectively): this agreement has implications for the applicability of laboratory findings to natural systems.

Résumé : Des données du U.S. Fish and Wildlife Service sont utilisées pour l'étude du lien entre la température de l'eau et la survie de saumoneaux quinnat (*Oncorhynchus tshawytscha*) de remonte automnale élevées en pisciculture, migrant par le delta des rivières Sacramento et San Joaquim, en Californie. Un modèle statistique conforme est présenté pour la libération des saumoneaux munis d'une micromarque magnétisée codée (MMC) dans le cours inférieur de la Sacramento et la récupération subséquente dans le delta, au moyen de chaluts pélagiques. Ce modèle considère la survie comme une fonction logistique de la température de l'eau et de la libération et la récupération subséquente de différents groupes de MMC comme des expériences distinctes de marquage et de recapture. La méthode des moindres carrés respondérés itérativement est utilisée pour ajuster le modèle aux données et on utilise la simulation pour établir des intervalles de confiance pour les paramètres ajustés. La température létale supérieure initiale déduite des données recueillies au moyen du relevé au chalut, grâce à cette méthode, se situe à 23,01 ± 1,08°C, avec un niveau de confiance de 95%. Cette valeur correspond aux résultats publiés d'expériences, obtenus dans des conditions contrôlées, donnant respectivement 24,3 ± 0,1 et 25,1 ± 0,1°C, pour le quinnat acclimaté à 10 et 20°C. Cette correspondance ouvre des possibilités quant à l'applicabilité aux systèmes naturels des résultats obtenus en laboratoire.
[Traduit par la Rédaction]

Introduction

For many years, the U.S. Fish and Wildlife Service (USFWS), in cooperation with the California Department of Fish and Game (CDFG) through the Inter-Agency Ecological Study Program, has conducted trawls for chinook salmon (*Oncorhynchus tshawytscha*) smolts near Chipps Island in the Sacramento – San Joaquin Delta of California during the main periods of smolt outmigration (USFWS

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1983–1992). The data arising from the Chipps Island trawls are used by USFWS and others to address a variety of questions about California's chinook salmon, such as smolt abundance, timing of outmigration, migration rates, and survival (Stevens et al. 1984; USFWS 1987; Kjelson et al. 1989).

An important part of these data consists of the recoveries of hatchery-reared fall-run smolts bearing coded-wire tags (CWTs) from a series of releases by USFWS and CDFG since 1978. These releases are made at a number of locations in the lower Sacramento River and northern delta specifically to provide information about smolt survival in the delta.

The usual treatment of these data has been as follows: an estimate is made of the survivorship associated with each individual release, the estimates are plotted against proposed explanatory variables (water temperature, smolt size, etc.), and a hypothesized survival curve is fitted through these points. Disagreements over the interpretation of the data have turned on the method used to estimate the individual survivorships and the functional form of the curve to be fitted (Kjelson et al. 1989; Baker et al. 1992).

This approach is reasonable and straightforward. It also has some limitations: it does not provide objective ways of assessing the extent to which a proposed survival function is consistent with the data, and it does not produce confidence bounds on fitted parameters that might be used to make informed policy decisions. Questions about goodness of fit and statistical uncertainty can only be formulated properly in the context of statistical models.

In this paper, we restrict our attention to the problem of estimating smolt survival as a function of water temperature, from trawl recoveries of CWT-marked smolts released at a single location. We show that a biologically reasonable model fits the data well enough to permit quantitative assessments of the uncertainty in the fitted parameters. The fitted values are shown to agree well with the results of laboratory studies.

Data

In this paper, r denotes the number of smolt release groups. For the ith release, $1 \le i \le r$, n_i is the number of smolts released, m_i is the number of smolts recovered, p_i is the trawl effort, and T_i is the water temperature at Ryde at the time of release, in degrees Celsius.

The data used in the models are those from the 15 releases in the lower Sacramento River at Ryde from 1983 through 1990 that are listed in Table 1. These data were assembled from USFWS (1983–1992) and Johnson and Longwill (1991). The smolts were all fall-run chinook salmon, reared at the Feather River Hatchery and released at Ryde in May or June. The average weight of these smolts ranged in different years from 5.15 to 9.40 g. Peak trawl recoveries at Chipps Island ranged from 2 to 5 d after release at Ryde.

Ryde is about 48 km upstream of Chipps Island, just below the last major distributary branching of the Sacramento River as it enters the delta. From each of the other release locations, there are alternate routes to Chipps Island and a variety of conditions to be found along the different routes. Smolts released at Ryde have only one direct route to Chipps Island (a second route, around Sherman Island via Three Mile Slough, is probably of minor importance), and survival along this route is likely to be less affected by factors other than water temperature than is survival through most other parts of the Delta. For this reason, the Ryde releases are commonly recognized as the most natural ones to consider when temperature is the primary variable of interest (Kjelson et al. 1989). Figure 1 shows the region of the delta under discussion.

What we are calling trawl effort is defined in USFWS reports as the ratio of the time spent in actual trawling to the total time interval covered by the surveys, multiplied by the ratio of the net width to the channel width. Although the USFWS reports do not always report the trawl effort, it is possible to recover it from the information that is reported. We will use the trawl effort as an estimate of the probability of capture; this assumption will be examined briefly later in this paper. The USFWS itself scrupulously refers to this quantity as simply an expansion factor, and to values calculated from it as survival indices.

The base model

All of our models begin with the assumption that the different CWT releases can be treated as independent mark–recapture experiments. For our first model, we treat each individual release as a binomial experiment, whose parameter is broken down into two components: the probability of survival from Ryde to Chipps Island, which we will take to be a logistic function $\phi(T_i)$ of water temperature T_i, and the probability of capture at Chipps Island, the known constant p_i. The parameters to be fitted are the location and scale parameters b_1, b_2 of the logistic function ϕ.

This corresponds to the likelihood function

$$L = \prod_1^r \pi_i$$

where

$$\pi_i = \pi(m_i \mid n_i, \phi_i, p_i) = \binom{n_i}{m_i} (p_i\phi_i)^{m_i} (1 - p_i\phi_i)^{n_i - m_i}$$

[1]

$$\phi_i = \phi(T_i) = \frac{1}{1 + e^{-b_1 - b_2 T_i}}$$

This is a generalized linear model with canonical link function, in the terminology of McCullagh and Nelder (1989). A model of this kind is completely specified by its mean and the dependence of the variance on the mean. In this case:

$$E[m_i] = p_i\phi_i n_i$$

[2]

$$V[m_i] = E[m_i] - \frac{1}{n_i} E[m_i]^2$$

The maximum likelihood estimate for (b_1, b_2) is easily found from Eq. 2 by the algorithm of iteratively reweighted least squares.

Table 1. Data for the release and recovery of selected coded-wire-tag groups of chinook salmon smolts released in the Sacramento River at Ryde, Calif. (From USFWS 1983–1992.)

i	Coded-wire-tag No(s).	Date of release	Average weight (g)	Temperature (°C), T_i	No. released, n_i	No. recovered, m_i	Trawl effort, p_i
1	06–62–23	5/20/83	5.89	16.1	92 693	95	0.000 833 24
2	06–42–09						
	06–62–29	6/13/84	5.15	18.9	59 998	37	0.000 880 98
3	06–62–35	5/11/85	5.82	18.9	107 161	88	0.001 066 49
4	06–62–48	5/30/86	5.34	23.3	101 320	74	0.001 123 63
5	06–62–55	4/29/87	5.79	19.4	51 103	46	0.001 058 99
6	06–62–58	5/2/87	6.21	17.8	51 008	47	0.001 071 42
7	06–31–01	5/3/88	8.40	17.2	52 741	106	0.002 138 11
8	06–31–02	5/6/88	8.56	16.1	53 238	146	0.002 142 50
9	06–62–63	6/22/88	8.25	23.9	53 961	46	0.002 131 17
10	06–31–03	6/25/88	8.72	23.3	53 942	39	0.002 126 47
11	06–31–12	5/3/89	7.00	16.7	51 046	65	0.001 070 05
12	06–31–07	6/2/89	9.40	19.4	50 601	26	0.001 070 47
13	06–01–14–01–02	6/16/89	7.83	22.8	51 134	8	0.000 977 82
14	06–31–20	5/9/90	5.04	20.6	51 878	87	0.001 036 47
15	06–31–22	5/31/90	6.87	18.3	50 837	67	0.001 057 73

A biologically natural alternative to the parameterization (b_1, b_2) of the survival curve is (LT50, α), where LT50 is the temperature at which the predicted survival is 0.50, and α is the slope of the survival function at T = LT50. We will report results in both forms.

For the data in Table 1, maximum likelihood estimation gives $b_1 = 15.89$, $b_2 = -0.6873$. Equivalently, LT50 = 23.12, $\alpha = -0.1718$.

The Pearson χ^2 for the fit is 104.5 with 13 degrees of freedom. The log-likelihood ratio statistic D, which is also approximately distributed as a χ^2 statistic with 13 degrees of freedom, is 103.4. Both of these values are very highly significant, indicating that the base model does not fit very well.

Table 2 shows the expected and observed numbers of trawl captures, with Pearson and deviance residuals. The residuals are plotted against water temperature in Fig. 2. Because there is no clear trend in the residuals, we do not attribute the lack of fit to a fundamental defect in the model structure, such as an inadequate choice of the functional form for ϕ. That is, the model's handling of temperature is acceptable, but the model is not flexible enough to account for all of the noise in the data from factors not included.

Overdispersion

The overdispersion of the data with respect to the base model is not necessarily a fatal defect; in fact, overdispersion is so common in models such as this that its absence would be more remarkable than its presence (cf. McCullagh and Nelder 1989, section 4.5.1).

A conventional way to deal with overdispersion in a situation like this is to simply inflate the variance by some constant σ^2. In this case, one would replace Eq. 2 by

$$E[m_i] = p_i \phi_i n_i$$

$$V[m_i] = \sigma^2 \left(E[m_i] - \frac{1}{n_i} E[m_i]^2 \right) \quad [3]$$

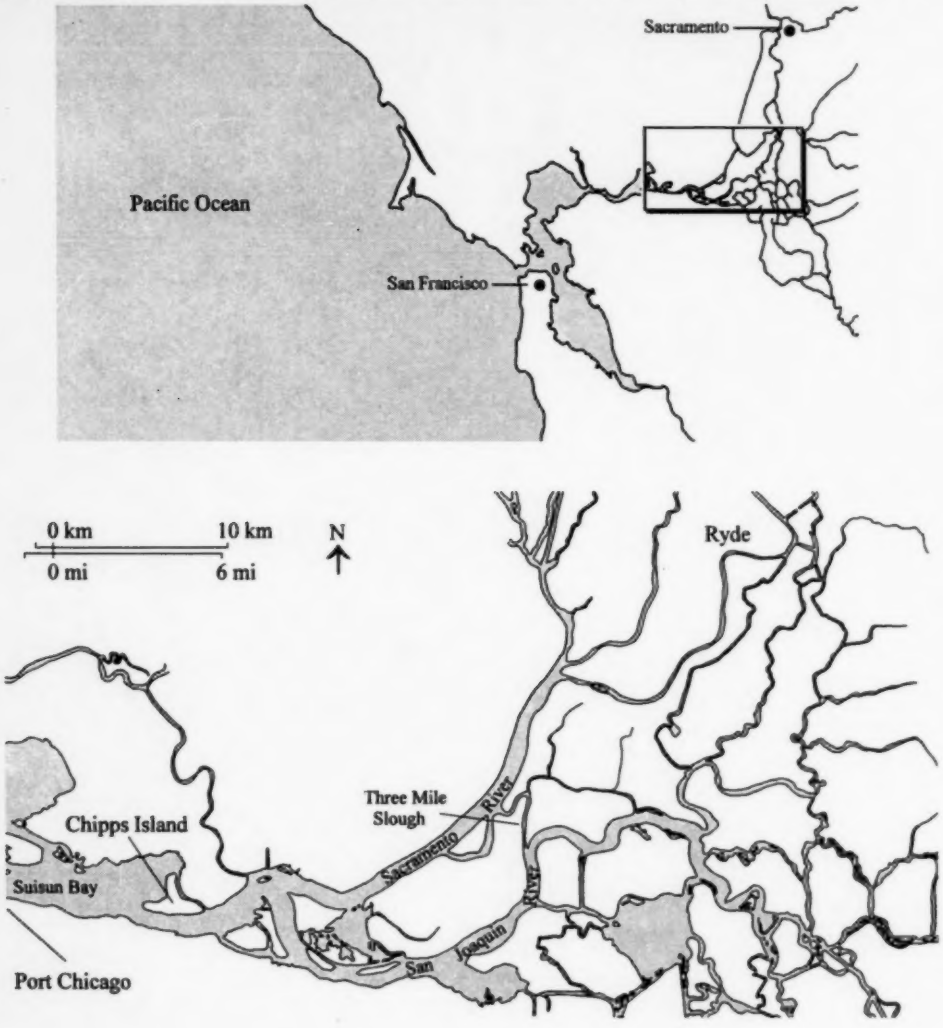
The maximum-likelihood estimate for (b_1, b_2) is not affected at all by the introduction of the dispersion parameter σ^2, so we are free to give σ^2 whatever value we want. In particular, we could force the model to have an acceptable chi-square fit simply by setting $\sigma^2 = \chi^2 \text{df}$, where χ^2 is the fit of the original model.

The main criticism one can make of this procedure is that it seems rather arbitrary. If a model does not fit the data, the model assumptions are inadequate in some way, and should at least be reexamined. After all, the fitted values of the model parameters will not be meaningful if the model itself has no relation to reality, regardless of how we assign confidence levels.

In fact, there is an extensive literature on the subject, which basically justifies using the unadorned model to estimate parameters like b_1 and b_2, and dealing with overdispersion as indicated above (see references in McCullagh and Nelder 1989; Burnham et al. 1987). Nevertheless, we prefer to tailor our approach to the specifics of our situation.

There are many possible sources of overdispersion in these experiments: the probability of survival surely depends on factors other than water temperature; fish from different release groups have different histories; and fish from the same release group recovered in different trawls have different histories. However, we believe that the most important uncertainty is in the capture probabilities p_i. It is clear from the nature of the experiment that these numbers could be in error by very large amounts. It is easy to imagine that smolts could have a preference for regions of the

Fig. 1. Study area in the north-central region of the Sacramento – San Joaquin Delta, California.



channel cross section that are especially likely or unlikely to be sampled in a particular trawl, or that they travel past Chipps Island in clumps that might or might not coincide with a trawl pass.

Furthermore, the data from some of the individual releases clearly point to errors in the capture probability estimates. In the first of the two 1990 releases, 51 878 smolts were released, of which 87 were recovered; even if the survival were 100%, the probability of recovering as many as 87 smolts, assuming that the probability of capture was really 0.001036, would be on the order of 10^{-5}.

On the other hand, there is evidence that the recovery probability estimates are not systematically too high or too low. Fish from the CWT groups released at Ryde are also recovered in the ocean fishery as adults; information about these recoveries is available through the Pacific States Marine Fisheries Commission. These recoveries can be used to generate estimates of delta smolt survival.

The CWT groups are recovered as 2-, 3-, 4-, and 5-year-olds (the nominal ages of fall-run chinook salmon are based on the calendar years in which spawning took place). By comparing the ocean recovery rates of 2 year olds from the Ryde groups with the ocean recovery rates for 2 year olds from groups of similar smolts released near Chipps Island at about the same time, it is easy to obtain estimates of survival (S) from Ryde to Chipps Island from individual releases. In fact, the closest release site to Chipps Island is Port Chicago, about 8 km downstream, so that what is being estimated is survival from Ryde to Port Chicago:

Table 2. Comparison of the trawl recoveries predicted by the fitted base model for the Ryde release groups with the corresponding actual trawl recoveries.

i	Expected recoveries	Actual recoveries	Pearson residuals	Deviance residuals
1	77	95	2.10	2.02
2	50	37	−1.86	−1.95
3	108	88	−1.96	−2.03
4	53	74	2.91	2.74
5	50	46	−0.58	−0.59
6	53	47	−0.86	−0.88
7	111	106	−0.46	−0.46
8	113	146	3.09	2.96
9	43	46	0.50	0.50
10	53	39	−1.95	−2.05
11	54	65	1.50	1.45
12	50	26	−3.41	−3.76
13	28	8	−3.78	−4.46
14	46	87	6.07	5.39
15	52	67	2.11	2.01

$$S_{\text{ocean}} = \frac{m_{\text{Ryde}} / n_{\text{Ryde}}}{m_{\text{PC}} / n_{\text{PC}}}$$

where n_{Ryde} is the number released at Ryde, n_{PC} is the number released at the Port Chicago, and m_{Ryde} and m_{PC} are the corresponding numbers recovered as 2 year olds in the ocean. These can be compared with simple estimates of survival from Ryde to Chipps Island for the same releases: $S_{\text{trawl}} = m_i/n_i p_i$ where n_i, m_i, and p_i are as defined earlier (cf. USFWS 1987).

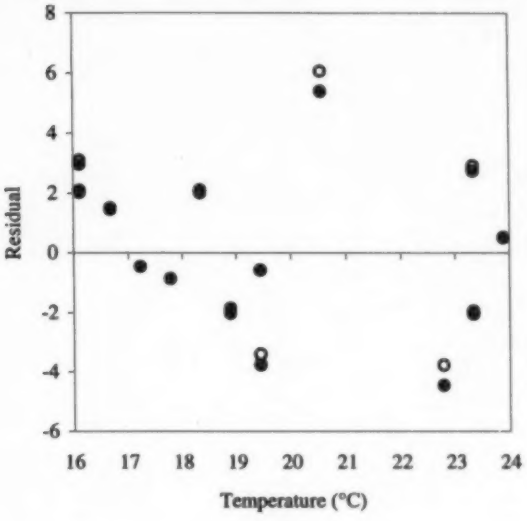
Survival from Chipps Island to Port Chicago should be high, because the distance between them is fairly small, so that S_{ocean}, S_{trawl} are essentially estimates of the same quantity. As there is no reason to expect both estimates to be biased in the same direction and to the same extent, each serves as a check on the other. Formal analysis confirms the impression of Fig. 3, that the hypothesis $S_{\text{ocean}} = S_{\text{trawl}}$ cannot be rejected at the 95% confidence level. We interpret this as evidence that the p_i can be used as estimates of the expected values of the true recovery probabilities (although the co-occurrences of ocean-based estimates greater than 1 with trawl-based estimates greater than 1 remains puzzling).

More information on the relationship between the trawl-recovery and ocean-recovery estimates can be obtained from the authors.

The relaxed model, the quasilikelihood estimator, and simulation

We modify the base model (Eq. 1) to allow for uncertainty in the capture probabilities by assuming that the capture probability P in the ith release is itself a random variable with mean p_i and variance $\rho^2 p_i^2$. Here ρ^2 is taken to be the same for all release groups. (Because the capture probabilities are necessarily non-negative, and we expect the errors in the p_i to be large, a multiplicative error structure seems called for; this leads to the assumption that the coefficient of variation, rather than the variance itself, is constant from release to release.) This gives

Fig. 2. Pearson (open circles) and deviance (solid circles) residuals for the fitted base model, plotted against water temperature.



$$\pi(m_i|n_i, \phi_i p_i) = \int_0^1 \binom{n_i}{m_i} (P\phi_i)^{m_i} (1 - P\phi_i)^{n_i - m_i} f_i(P)\, dP \quad [4]$$

$$\phi_i = \phi(T_i) = \frac{1}{1 + e^{-b_1 - b_2 T_i}}$$

where f_i is the density for P. We will call this the relaxed model.

Because we have not specified the distribution f_i, this is not yet a well-defined likelihood. No matter what distribution we use, however, we will always have

$$E[m_i] = p_i \phi_i n_i$$

$$V[m_i] = E[m_i] + \left(\frac{n_i - 1}{n_i}\rho^2 - \frac{1}{n_i}\right) E[m_i]^2 \quad [5]$$

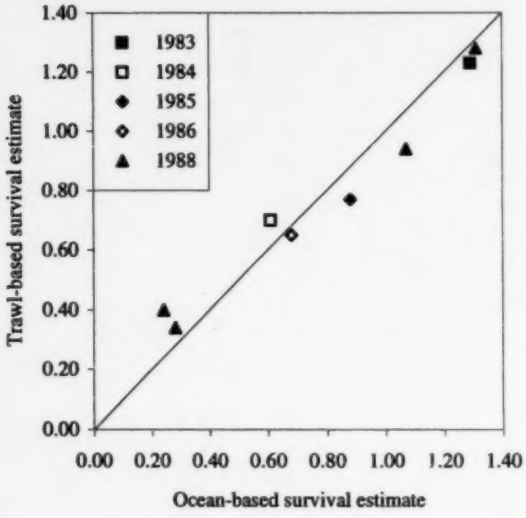
equivalently,

$$E[m_i] = E[m_i|P = p_i],$$

$$\frac{V[m_i]}{E[m_i]^2} = \frac{V[m_i|P = p_i]}{E[m_i|P = p_i]^2} + \frac{n_i - 1}{n_i}\rho^2.$$

If the π_i were in a suitable exponential family, this would be all the information necessary to find the maximum-likelihood estimate for (b_1, b_2) by iteratively reweighted least squares. This algorithm is in any case a perfectly legitimate estimator, that one would expect to inherit some of the properties of a genuine maximum-likelihood

Fig. 3. Two methods of estimating smolt survival from Ryde to Chipps Island. The diagonal line (trawl-based survival = ocean-based survival) is provided for reference.



estimator. We will refer to this as the quasilikelihood estimator, for reasons to be discussed in the next section.

We are interested not only in the parameter estimates themselves, but in statistical properties of the estimator such as bias and variance. The conventional way to assign confidence intervals to the parameter estimates is by the δ-method. In the case of generalized linear models fitted by iteratively reweighted least squares, the covariance matrix emerges naturally from the algorithm; when a model that is not necessarily of this form is fitted by the iteratively reweighted least-squares algorithm, the algorithm gives the covariance matrix asymptotically. In either case, the estimators are approximately unbiased and asymptotically normal (McCullagh and Nelder 1989).

However, maximum-likelihood estimators can be very far from either unbiased or normal when the number of samples is not large. In any case, these compromises are entirely unnecessary. For any particular choice of f_i, the properties of the quasilikelihood estimator can be determined to any desired accuracy by simulation.

We will consider two simple examples, the uniform distribution:

$$f_i(P) = \begin{cases} \dfrac{1}{2w}, & \text{if } |P - p_i| < w \\ 0, & \text{otherwise} \end{cases}$$

where

$$w = p_i\sqrt{3\rho^2}$$

and the triangular distribution

$$f_i(P) = \begin{cases} \dfrac{1}{w}\left(1 - \dfrac{1}{w}|P - p_i|\right), & \text{if } |P - p_i| < w \\ 0, & \text{otherwise} \end{cases}$$

where

$$w = p_i\sqrt{6\rho^2}$$

The largest value of ρ^2 consistent with the uniform distribution is one-third, and the largest value consistent with the triangular distribution is one-sixth. Notice that the uniform distribution has the largest variance of any unimodal distribution symmetric about p_i, and so sets an upper limit on the amount of extra variation that can be reasonably attributed to uncertainty in p_i. Confidence estimates based on this distribution should therefore be conservative.

We have defined a model (or at least a family of models) and a fitting procedure. It still remains to choose a value for ρ^2. We have no good basis for selecting a value a priori. Not only do we lack a suitable understanding of the trawl capture process, but the parameter is absorbing extra variation associated with ϕ and with the approximation of the trawl recovery as a simple binomial process. There are methods for fitting ρ^2 formally as a model parameter (McCullagh and Nelder 1989), but for a data set of this size we find it more appropriate to simply pick a value that results in a reasonable model fit. We have followed the usual practice of forcing the Pearson χ^2-statistic of the fit to equal the degrees of freedom (Williams 1982).

For the data in Table 1, the fitting procedure described above produced the estimate $\rho^2 = 0.1503$. This value for ρ^2 seems plausible to us. It is close to the ρ^2 for the maximally broad triangular distribution, and comfortably within the range of ρ^2-values that are consistent with the derivation of the model.

For this value of ρ^2, the fitted parameters are $b_1 = 15.56$, $b_2 = -0.6765$, so that LT50 = 23.01 and $\alpha = -0.1691$.

Confidence intervals and bias for b_1, b_2, LT50, and α were estimated by simulation: the model (4) was used with both the uniform and triangular distributions for f_i to generate 5000 data sets each, assuming the values for ρ^2, b_1, and b_2 given above. Each simulated data set was fitted to the model (holding ρ^2 constant), yielding 10 000 pairs (b_{1k}, b_{2k}).

The mean, standard deviation, and bias of these data, and some order statistics, are shown in Table 3. Standard formulas show that the mean and standard deviation are determined by the simulation to within 2% at the 95% confidence level. The quasilikelihood estimator for LT50 is seen to be essentially unbiased, confirming the naturalness of this quantity as a model parameter. The shortest 95% confidence intervals were 21.96°C < LT50 < 24.10°C for the uniform distribution and 22.59°C < LT50 < 23.41°C for the triangular distribution. The corresponding symmetric 95% intervals were 21.93°C < LT50 < 24.08°C and 22.60°C < LT50 < 23.42°C, respectively.

The results of the simulation are shown more vividly in Fig. 4. For each model, one point has been plotted at a randomly chosen temperature on each of the 5000 fitted survival curves, to give some feeling for the shapes of the confidence surfaces.

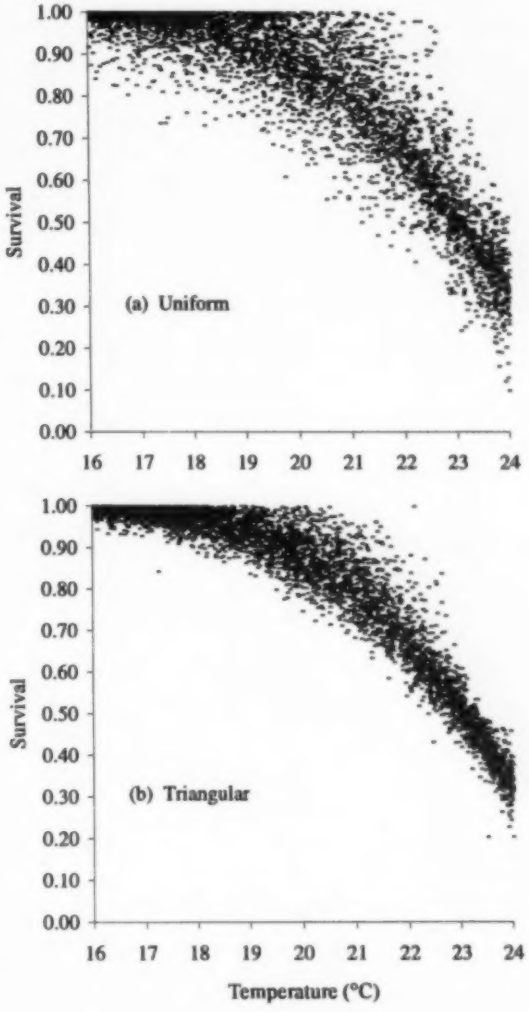
The quasilikelihood-generating model

Our goal in this section is to clarify just what the quasilikelihood estimator of the preceding section is maximizing.

Table 3. Statistical properties of the quasilikelihood estimators, determined by simulation with respect to two models of capture probability.

	Canonical parameters		Natural parameters	
	b_1	b_2	LT50	α
Fitted	15.56	−0.6765	23.01	−0.1691
Uniform				
Mean	18.65	−0.8080	23.06	−0.2020
SD	10.18	0.4356	0.57	0.1089
Bias	3.08	−0.1315	0.05	−0.0329
P1	5.72	−2.6166	21.64	−0.6542
P2.5	7.40	−2.0770	21.95	−0.5193
Q1	13.09	−0.8957	22.85	−0.2239
Median	15.80	−0.6880	23.03	−0.1720
Q3	20.70	−0.5722	23.26	−0.1430
P97.5	47.97	−0.3168	24.10	−0.0792
P99	60.60	−0.2352	24.63	−0.0588
Triangular				
Mean	16.80	−0.7291	23.01	−0.1823
SD	5.06	0.2163	0.21	0.0541
Bias	1.23	−0.0526	0.01	−0.0132
P1	10.09	−1.5716	22.47	−0.3929
P2.5	10.75	−1.3101	22.57	−0.3275
Q1	13.62	−0.8028	22.88	−0.2007
Median	15.62	−0.6810	23.02	−0.1703
Q3	18.54	−0.5941	23.16	−0.1485
P97.5	30.32	−0.4690	23.40	−0.1172
P99	36.23	−0.4414	23.48	−0.1103

Fig. 4. Distributions of quasilikelihood estimates of smolt survival from Ryde to Chipps Island, for the fitted model, assuming that the probability of capture is drawn from (*a*) the uniform distribution and (*b*) the triangular distribution.



From a practical point of view, the question is moot, in that the simulations described there establish completely rigorous confidence regions for the estimated parameters. This section can be skipped by readers who are primarily interested in the biological results.

Quasilikelihood theory was developed to deal with situations in which one has some (usually empirical) information about the relationship between the expected value and variance of a quantity, over a series of similar experiments, but not about the statistical mechanisms that give rise to this relation and, therefore, no way to construct a likelihood function. In such a situation, one can construct a function called a quasilikelihood, which turns out to have many of the properties of a true likelihood function arising from a generalized linear model. In particular, the method of iteratively reweighted least squares can be used to maximize the quasilikelihood, and much of the asymptotic theory of maximum likelihood estimation carries over to maximum quasilikelihood (McCullagh and Nelder 1989).

Our case is rather different, in that we have the definite model (4) in mind, which is only incomplete in that we are trying to avoid committing ourselves to a particular form for the functions f_i.

If there were a suitable exponential family distribution having the same mean and variance as Eq. 4, the quasilikelihood estimate would be exactly the maximum likelihood estimate for this distribution. Unfortunately, it is not hard to show that no such distribution exists. The obstacle here turns out to be the requirement that the distribution is supported on the integers from 0 to n. If this condition is relaxed to require only that the distribution be supported on non-negative integers, there is a (unique) exponential family distribution with the desired properties:

$$\binom{n_i/\gamma_i}{m_i}(\gamma_i p_i\phi_i)^{m_i}(1-\gamma_i p_i\phi_i)^{n_i/\gamma_i-m_i}, \quad \text{for } 0<\gamma_i<1$$

$$[6]\qquad \pi(m_i|n_i,\phi_i,p_i)=\frac{(p_i\phi_i n_i)^{m_i}}{m_i!}e^{-p_i\phi_i n_i}, \quad \text{for } \gamma_i=0$$

$$\binom{-n_i/\gamma_i + m_i - 1}{m_i}(-\gamma_i p_i\phi_i)^{m_i}(1-\gamma_i p_i\phi_i)^{n_i/\gamma_i - m_i}, \quad \text{for } \gamma_i < 0$$

where $\gamma_i = 1 - (n_i - 1)\rho^2$.

Except for a constant factor, this turns out to be identical to the quasilikelihood function constructed from Eq. 5, so that it reasonable to call Eq. 6 the quasilikelihood generating model.

Because the number of smolts in each release ($\approx 10^4$, 10^5) is very much larger than the typical number recovered ($\approx 10^1$, 10^2), it would have been quite reasonable to model the underlying survival–capture process as a Poisson process. After all, the binomial model is also only an approximation (for example, smolts from one release are actually recovered over several trawls), and it would be difficult to argue convincingly that it is a better one than the Poisson in this case. If we imitate the development of the previous section, beginning from the Poisson model, things work out pretty much as before. The mean and variance functions of the relaxed model become

$$[7] \quad \begin{aligned} E[m_i] &= p_i\phi_i n_i \\ V[m_i] &= E[m_i] + \rho^2 E[m_i]^2 \end{aligned}$$

and the quasilikelihood-generating distribution takes the form:

$$= \frac{(p_i\phi_i n_i)^{m_i}}{m_i!} e^{-p_i\phi_i n_i}, \quad \text{for } \gamma_i = 0$$

$$[8] \quad \pi(m_i|n_i, \phi_i, p_i) = \binom{-n_i/\gamma_i + m_i - 1}{m_i}(-\gamma_i p_i\phi_i)^{m_i}(1-\gamma_i p_i\phi_i)^{n_i/\gamma_i - m_i}, \quad \text{for } \gamma_i < 0$$

where $\gamma_i = -n_i\rho^2$ (so the first case of Eq. 6 never arises). These equations are identical to Eqs. 5 and 6 except for obviously negligible terms of order $1/n_i$.

The second (negative binomial) distribution of Eq. 8, however, can also be exhibited as the model that results from the Poisson base model when the parameter p_i is replaced by a gamma variate with mean p_i and variance $\rho^2 p_i^2$. That is, the quasilikelihood estimate is indeed a maximum-likelihood estimate for a perfectly natural model. Our only reason for preferring the language of quasilikelihood is that the maximum-likelihood interpretation depends very delicately on making the right approximations.

Discussion

We have shown that a simple and natural model of smolt survival can be fit to the data. This model predicts mean smolt survival at a given temperature to about 10% at the 95% confidence level (cf. Fig. 4).

Taking the most conservative error bounds, we have estimated that chinook salmon released at Ryde and migrating to Chipps Island experience 50% mortality at 23.01 ± 1.08°C. It is interesting to compare this estimate of survival under natural conditions with the results of laboratory studies.

Laboratory studies of the direct effects of high temperatures on animal survival have been conducted in two different ways: the method of abrupt transfer and the method of slow heating (Kilgour and McCauley 1986). These result in somewhat different measures of lethality. For our purposes we will regard the upper incipient lethal temperature (UILT) found in abrupt transfer experiments as comparable with the LT50 of the fitted model. We will regard the temperatures at which given fractions of the sample are lost in slow heating experiments as comparable with the temperatures at which these same losses are predicted by the model. In both kinds of experiments, the results depend on the temperature to which the animals were acclimatized.

The classic abrupt transfer experiments involving chinook salmon are those of Brett (1952):

	Brett (1952)				Fitted
Acclimation (°C)	10	15	20	24	—
UILT	24.3±0.1	25.0±0.1	25.1±0.1	25.1±0.1	23.01±1.08

We regard this as a reasonable agreement.

The temperatures predicted by the fitted model to result in 10, 50, and 90% mortality are also consistent with the results of several slow-heating experiments reproduced in the survey of Houston (1982):

	Houston (1982)						Fitted
Acclimation (°C)	10	10	11	13	18	20	—
10% loss	22.9	20.5	23.0	19.5	20.0	23.8	19.76
50% loss	—	—	23.5	—	—	24.7	23.01
90% loss	24.5	23.5	23.8	23.0	23.5	24.8	26.26

The laboratory studies cited above examine the effects of temperature alone. In the natural environment, however, it may be difficult or impossible to separate the direct effects of temperature from indirect effects on the ability of salmon to survive other threats, such as predation and disease. It is reasonable to inquire about the magnitude of these indirect effects.

The UILTs found by Brett for salmon acclimatized to 15°C and above are about 2°C higher than the LT50 found here. In addition, the range of temperatures at which significant temperature-related mortality occurs is greater in the fitted model than in any of the laboratory studies referred to above. Both of these observations would be consistent with the presence of significant indirect effects of temperature on survival in the delta. If the possibility of differences in temperature tolerance between Central Valley salmon stocks and the more northerly stocks used in the laboratory studies is considered, there may be even more room for indirect temperature effects. On the other hand, the

model makes no provision for possible sources of mortality independent of temperature. If mortality from such sources could be accounted for separately, the LT50 associated with the remaining mortality would probably be higher.

Our analysis shows that direct effects of high temperature are sufficient to explain a large part of the smolt mortality actually observed in the delta. In particular, the observed LT50 of 23.01 ± 1.08°C is remarkably consistent with the results of controlled experiments. This reaffirms the relevance of laboratory findings to natural systems.

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Kinetics of excess (above background) copper and zinc in *Hyalella azteca* and their relationship to chronic toxicity

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Abstract: One-week-long metal uptake experiments with *Hyalella azteca* are potentially a powerful tool for rapidly assessing the impact of copper or zinc in contaminated water samples. Copper and zinc concentrations in whole body *Hyalella* are independent of body size for both control and metal-exposed amphipods. Uptake rates are rapid for both metals, but copper concentrations in *Hyalella* during continuous exposure peak at about 1 week, then gradually decline back towards control levels at an apparent rate of 0.039 d^{-1}. *Hyalella* can, therefore, control body copper concentrations during exposure to elevated copper, but only gradually and after long exposure periods. Depuration rates for copper and zinc (0.16 and 0.68 d^{-1} respectively) are approximately five times slower than the apparent depuration rates during uptake, assuming a simple diffusion model. Uptake at increasing water concentrations results in saturation for both metals with maximum accumulations of 3.6 μmol/g dry weight above background. A mathematical model including saturation kinetics satisfactorily explains the more rapid approach to equilibrium observed during uptake than during depuration. Significant chronic mortality occurred at metal exposures that resulted in predicted accumulations of 1.8 μmol Cu/g or 1.3 μmol Zn/g above background after 1 week of uptake.

Résumé : Des expériences d'une semaine sur l'absorption de meetaux par *Hyalella azteca* pourraient être un bon moyen d'évaluer rapidement l'impact du cuivre ou du zinc dans des échantillons d'eau contaminée. Les concentrations de cuivre et de zinc dans l'ensemble du corps d'*Hyalella* sont indépendantes de la taille du corps tant chez les amphipodes témoins que chez les spécimens exposés aux métaux. L'absorption est rapide pour les deux métaux mais les concentrations de cuivre dans *Hyalella* pendant une exposition continue culminent à environ une semaine, après quoi elles redescendent à des niveaux de contrôle, à un rythme d'environ 0,039 d^{-1}. *Hyalella* peut donc limiter les concentrations de cuivre qu'elle absorbe, à des niveaux d'exposition élevés, mais seulement de manière graduelle et après une longue exposition. Les taux de dépuration, pour le cuivre et le zinc (respectivement 0,16 et 0,68 d^{-1}) sont environ cinq fois plus lents que les rythmes apparents de dépuration pendant l'absorption, sur la foi d'un modèle de diffusion simple. L'absorption, à des concentrations croissantes dans l'eau, aboutit à la saturation pour les deux métaux, avec des accumulations maximales de 3,6 μmol/g, matières sèches, au-dessus de la concentration naturelle. Un modèle mathématique comportant la cinétique de saturation explique de manière satisfaisante la montée vers l'équilibre observée pendant l'absorption, plus rapide que pendant la dépuration. Une mortalité chronique importante a été enregistrée à différentes expositions; on a constaté, comme prévu, des accumulations de 1,8 μmol de Cu/g et de 1,3 μmol de Zn/g, au-dessus de la concentration naturelle, après une semaine d'absorption.

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Introduction

Metal accumulation is potentially one of the most valuable tools for identifying and quantifying the impact of metals in aquatic environments. Toxicity is strongly affected by water chemistry and particulate matter. Metal accumulation, however, is affected by the same parameters that affect toxicity. Contaminant (whether metal or organic) uptake at the site of toxic action would normally be expected to occur before toxicity appears. Assuming that the total body concentration is roughly proportional to the concentration of a contaminant at the site of toxic action, toxicity should be more constant if expressed on a body-concentration, rather than water-concentration, basis (e.g., Connolly 1985; Landrum et al. 1992; McCarty and Mackay 1993). This has been demonstrated, for example, for cadmium toxicity to *Hyalella azteca* in waters with varying degrees of complexing capacity, and in the presence and absence of sediments (Borgmann et al. 1991). Unfortunately, most toxicity experiments have related toxicity only to metal concentrations in water, and not metal accumulated in the body. More data on the relationship between metal accumulation and toxicity are needed.

Amphipods have been suggested as useful biomonitors of copper and zinc contamination in the environment (Rainbow et al. 1989; Moore et al. 1991). Many decapods can regulate copper and zinc (e.g., White and Rainbow 1982; Bryan et al. 1986), but in more primitive crustaceans, including some amphipods, copper and zinc either are not, or are more poorly, regulated (Rainbow and White 1989). This makes amphipods particularly appealing as biomonitors of environmental metal availability. The amphipod *H. azteca* is being increasingly used in freshwater sediment toxicity evaluations in North America, and several standard sediment bioassays have been proposed (Borgmann and Munawar 1989; Ingersoll and Nelson 1990; American Society for Testing and Materials 1991). Furthermore, its high sensitivity to many contaminants (Borgmann et al. 1989; Ankley et al. 1991), and its widespread occurrence in North America, makes *Hyalella* a prime candidate for use as an indicator of metal contamination.

Chronic toxicity of zinc to *Hyalella* occurs when body concentrations reach approximately twice that of control amphipods, but copper concentrations in control amphipods and those exposed to copper for 10 weeks do not differ (Borgmann et al. 1993). However, copper is elevated in *Hyalella* after only 1 week of exposure, suggesting *Hyalella* gradually develops an ability to regulate internal copper during prolonged exposure. Significant accumulation occurs at concentrations below those causing chronic toxicity (Borgmann et al. 1993). This suggests that copper and zinc body concentrations, following short-term uptake, can be useful predictors of chronic toxicity. However, a detailed understanding of the uptake and depuration kinetics of these metals is required to correctly interpret metal uptake measurements and to design appropriate bioaccumulation tests. It is important to know, for example, if uptake is affected by body size (e.g., Rainbow and Moore 1986), and how long an exposure period is required for body concentrations to equilibrate with media. We, therefore, measured the uptake and depuration rates of copper and zinc in *Hyalella*, and determined the effect of body size and water concentration on excess metal (i.e., above background); the significance of background copper and zinc concentrations in *Hyalella* is discussed in the companion paper (Borgmann and Norwood 1995). Metal measurements were fitted to a kinetic model to ensure that our conclusions were internally consistent. Finally, the results were compared with previous chronic toxicity studies to provide an estimate of the lowest body concentration at which statistically significant mortality occurs.

Methods

Amphipods were cultured as described in Borgmann et al. (1989). Cultures and experimental animals were kept in Burlington city tap water (originating from Lake Ontario, hardness 130 mg/L, alkalinity 90 mg/L, pH 7.9–8.6) in an incubator at 25°C with a 16 h light : 8 h dark photoperiod. Water was changed in the cultures weekly, and young amphipods were removed at the same time. This provided a continuous supply of amphipods of known age for metal uptake measurements. Uptake experiments were usually initiated with 4- to 6-week-old amphipods, except as indicated (e.g., in experiments to determine the effect of body size on uptake). Experiments were performed in 250 mL of medium in 500-mL Erlenmyer flasks with loose fitting glass covers and one 5 × 10 cm piece of presoaked cotton gauze as substrate, unless otherwise indicated. The animals were placed in fresh flasks with renewed water, metals, and 5 mg of Tetra-Min fish food flakes once a week. Additional food was added during the week as required. These conditions were the same as those previously used to measure metal toxicity (Borgmann et al. 1991, 1993).

Unless otherwise indicated, amphipods were exposed to 0.79 μM (50 μg/L) copper and (or) 4.6 μM (300 μg/L) zinc. These are approximately equitoxic concentrations, because both 0.88 μM (56 μg/L) copper and 4.9 μM (320 μg/L) zinc resulted in roughly 50% survival relative to controls after 6 weeks of exposure; in each case, the next lower concentrations (0.50 μM (32 μg/L) copper and 2.75 μM (180 μg/L) zinc) were the lowest concentrations resulting in statistically significant chronic mortality (Borgmann et al. 1993). Water concentrations were measured in all experiments; mean measured concentrations at the above nominal exposure concentrations with water renewal once a week ranged from 93 to 96% of nominal for copper and from 85 to 97% of nominal for zinc. At selected time intervals amphipods were removed from experimental flasks, dried at 60°C in polypropylene cryovials, digested by addition of 25 μL of 70% nitric acid, and allowed to sit for 1 week. Then 20 μL of 30% hydrogen peroxide was added, followed after 24 h by 1 mL of double-distilled water. Most amphipods weighed from 0.2 to 0.8 mg dry weight and were digested individually. Smaller amphipods were pooled, with as many as 19 animals used per digestion for the smallest sizes (0.02 mg dry weight) in the body size experiments. Digested samples were analyzed for copper and zinc on a Varian SpectraAA 400 graphite furnace atomic absorption spectrophotometer with Zeeman background correction. Copper was measured in a partition tube without modifier; zinc was analyzed using a platform and ammonium phosphate modifier. Measured

copper and zinc concentrations in TORT-1 lobster hepatopancreas reference material (National Research Council, Canada), using the same analytical procedure, were 400 ± 5 μg Cu/g (mean ± SE, n = 27) and 188 ± 10 μg Zn/g (n = 28), compared with certified values of 439 μg Cu/g and 177 μg Zn/g.

Statistical analysis of body concentration data were performed by ANOVA using SYSTAT 5.0, with comparisons between treatments made by the Tukey method. Fitting of uptake and depuration curves was done by least squares. All body concentration data were logarithmically transformed before statistical analysis or least squares fitting to equalize variances.

Theory

The following terms are defined:

- C_w metal concentration in water
- C_{TB} total metal concentration in the body of *Hyalella*
- C_{Bk} background (control) metal concentration in *Hyalella*
- C_{Xs} $C_{TB} - C_{Bk}$ = excess (above background) metal concentration in *Hyalella*
- C_{X0} initial excess metal concentration (C_{Xs} at $t = 0$) in previously contaminated *Hyalella*
- C_{Xmax} maximum value of C_{Xs} obtained in *Hyalella* when fully saturated with metal
- k_u uptake rate of metal from water into *Hyalella*
- k_e elimination rate of metal by *Hyalella*
- k $k_uC_w + k_e$
- k_r rate of decrease in C_{Xs} with time during long-term exposure
- W dry body mass of *Hyalella*
- g $d(W)/(W \times dt)$ = instantaneous growth rate of *Hyalella*
- B_{Xs} excess metal on a total body burden basis ($C_{Xs}W$)
- B_{X0} initial excess metal on a total body burden basis ($C_{X0}W$).

The relationship among metal uptake, metal concentration in the water, and exposure time can be modelled on the assumption that C_{Xs} represents metal bound to the organism at selected sites that can be saturated. Binding at these sites is reversible. In contrast, C_{Bk} is assumed to represent irreversibly bound metal and is more or less constant (i.e., copper and zinc are essential metals and background metal is integrally incorporated in essential biochemical components (Borgmann and Norwood 1995)). The rate of change in the concentration of metal in the body is then given by

$$[1] \quad d(C_{TB})/dt = d(C_{Xs})/dt = k_u(C_{Xmax} - C_{Xs})C_w - k_eC_{Xs} - gC_{Xs} = k_uC_{Xmax}C_w - (k_uC_w + k_e + g)C_{Xs}$$

where $C_{Xmax} - C_{Xs}$ is the concentration of "free" metal-binding sites. The apparent reduction in C_{Xs} due to growth dilution is proportional to g.

Alternatively, changes in metal concentration can be described on a total body burden basis. Using the definitions of g and B_{Xs} given above, Eq. 1 gives

$$[2] \quad d(B_{Xs})/dt = k_uC_{Xmax}C_wW - (k_uC_w + k_e)B_{Xs}$$

where W is a function of time.

Short-term metal uptake

Equation 1 can be integrated exactly if g is constant. This is not the case for *Hyalella*. For this amphipod, body mass is approximately linearly related to time, resulting in a decreasing relative growth rate with increasing time and mass. An approximate solution to Eq. 1 is obtained over short intervals during which little growth occurs (i.e., $d(W)/dt \approx 0$) by setting $g = 0$. Integration of Eq. 1 then yields

$$[3] \quad C_{TB} \approx C_{Bk} + \frac{k_uC_{Xmax}C_w}{k_uC_w + k_e}(1 - \exp(-(k_uC_w + k_e)t)) + C_{X0}(\exp(-(k_uC_w + k_e)t))$$

where C_{X0} is greater than 0 only if animals have been exposed to high concentrations of the metal in water before $t = 0$. If $C_{X0} = 0$, then Eq. 3 can be simplified into

$$[4] \quad C_{TB} \approx C_{Bk} + C'_{Xmax}(1 - \exp(-kt))$$

where $C'_{Xmax} = k_uC_{Xmax}C_w/(k_uC_w + k_e)$ is constant at constant C_w. Equation 4 is used below for describing short-term (up to 4 day) metal uptake.

Long-term metal uptake during continuous exposure

Theoretically, Eq. 4 also provides a reasonably good description of long-term uptake during continuous exposure if $k >> g$. Under these conditions, C_{TB} reaches a maximum and becomes constant before a major change in body mass occurs. Copper concentrations during long-term exposure, however, gradually decline (see below). To describe this, an additional term (k_r) has been added to Eq. 4, giving

$$[5] \quad C_{TB} = C_{Bk} + C'_{Xmax}(1 - \exp(-kt))\exp(-k_rt)$$

Unlike Eq. 4, which is derived theoretically, Eq. 5 is defined empirically. A gradual return of C_{TB} to background concentrations could occur by many mechanisms (e.g., changes in k_u, k_e, or C_{Xmax}), and our data are insufficient to discriminate among these.

Metal depuration when $C_w = 0$

Equation 2 can be integrated exactly if $C_w = 0$. Upon integration, and using $C_{TB} = B_{Xs}/W + C_{Bk}$, this gives

$$[6] \quad C_{TB} = C_{Bk} + \frac{B_{X0}\exp(-k_et)}{W}$$

The application of Eq. 6 does not depend on the assumption that growth is negligible and can be applied to any growth curve. Equation 6 is used below for describing metal depuration for time periods of up to 2 weeks.

Results

Uptake and depuration experiments were performed using equivalent conditions, exposure times, and numbers of animals for experiments with both copper and zinc. For brevity, figures are usually shown for only the copper data; figures for the zinc data are similar, with similar variability, although computed rate constants differ somewhat.

Fig. 1. Copper accumulated by *H. azteca* exposed to 0 (△) or 0.79 (■) μM Cu for 1 week. The lower body size limit is 0.02 mg.

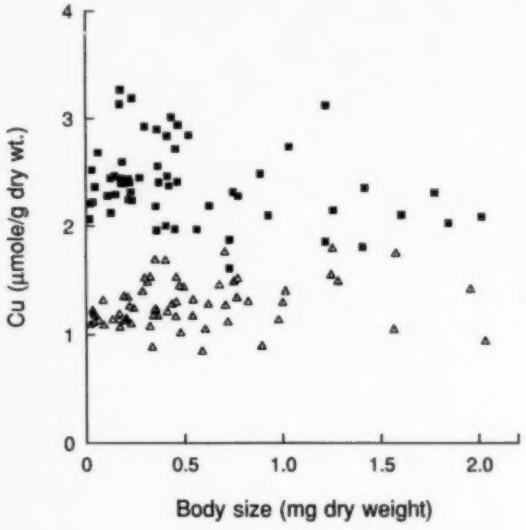
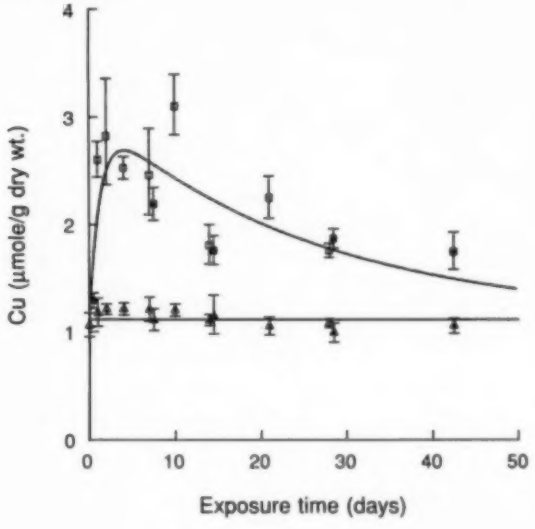


Fig. 2. Copper accumulated by *H. azteca* exposed to 0 (△ and ▲) or 0.79 (□ and ■) μM Cu for 1–42 days. Data for two separate experiments (open and solid symbols) are plotted together. The data points for the second experiment (▲ and ■) are shifted one-half day to the right for clarity. Vertical bars indicate ±1 SE; each point is the mean of 3–16 measurements (usually 6 for control and 5 for copper exposed). The mean control copper concentration is shown as a horizontal line. The curve was fitted by least squares to log-transformed data using Eq. 5 in the text.



Effect of body size (dry mass) on copper and zinc in *Hyalella*

The effect of body size, measured as dry mass, is one of the first parameters that needs to be investigated because a strong body size effect requires that body concentrations be normalized to a standard size organism whenever body size varies significantly (Rainbow and Moore 1986). Two experiments were performed, several months apart. The first involved measurement of copper and zinc in control animals maintained together for several weeks in a single aquarium to ensure equivalent exposure concentrations. The second involved exposure of all sizes together for 1 week in 2.5-L Pyrex jars with either no metal addition, copper or zinc singly, or both metals together.

Both copper and zinc concentrations changed only minimally with changes in body size in both experiments and for both control and exposed animals, even over a 100-fold range in body size (0.02–2 mg dry weight). Measured concentrations between the largest and smallest animals overlapped considerably. Data for copper in controls and copper-exposed amphipods are shown in Fig. 1; data for zinc are similar. If all data for each metal, after log transformation, are pooled into a single linear regression, using dummy variables to adjust for differences in absolute body concentrations between experiments and treatments, the overall estimate of the exponent b (±95% confidence limits) in the relationship: body concentration = constant × (body mass)^b is 0.006 ± 0.020 for copper (n = 257) and 0.023 ± 0.029 for zinc (n = 244). Neither regression is statistically significant. For all practical purposes, therefore, when dealing with amphipods of roughly similar sizes (e.g., within a factor of 10), the body size effect can be ignored. This greatly simplifies further analysis and negates the need for expressing copper or zinc concentrations relative to a standard size amphipod.

Copper uptake as a function of time

The accumulation of copper in *Hyalella* exposed to 0.79 μM Cu for 4–6 weeks demonstrated a very rapid accumulation within the first day of exposure, followed by a gradual decrease in body copper after the first week (Fig. 2). Copper in control animals remained fairly constant. Because of the very rapid rise in copper concentration, an additional experiment was conducted to determine the initial rate of uptake of copper. The short-term uptake data (Fig. 3) were fitted by nonlinear least squares regression to Eq. 4, giving an estimate for k of 0.73 d^{-1} (Table 1). Using this estimate of k, the long-term uptake data (Fig. 2) were then fitted to Eq. 5, giving k_r = 0.039 d^{-1}. The decrease in copper concentrations with prolonged exposure is statistically significant (95% confidence interval for k_r does not include 0; Table 1), indicating that *Hyalella* is capable of gradually reducing excess copper back to control concentrations. After 10-week exposure, a k_r of 0.039 would result in an excess copper concentration (i.e., copper above background) of only 6.5% of the original excess copper concentration, explaining why no relationship was observed between copper exposure and body concentration in previous 10-week exposures (Borgmann et al. 1993).

The data in Fig. 2 demonstrate that *Hyalella* is capable of regulating copper, although the process is not instantaneous and requires many weeks before concentrations

Fig. 3. Copper accumulated by *H. azteca* exposed to 0.79 μM Cu for up to 4 days. Vertical bars indicate ±1 SE; each point is the mean of 20 measurements. The curve was fitted by least squares to log-transformed data using Eq. 4 in the text.

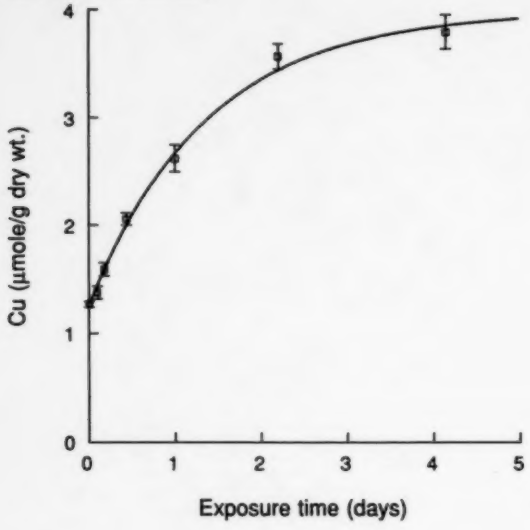
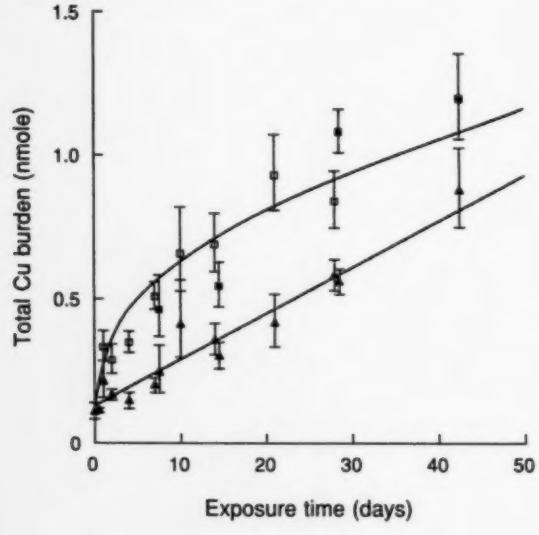


Fig. 4. Total body burden (concentration × body mass) accumulated by *H. azteca* exposed to 0 (△ and ▲) or 0.79 (□ and ■) μM Cu for up to 42 days. The lines are equivalent to the lines in Fig. 2 multiplied by (0.116 + 0.0143 days), the regression of dry weight against time. Error bars are larger than in Fig. 2 because they include variation in individual weights as well as variation in body concentrations.



approach background levels. The reduction in copper concentration is not associated with a net excretion of copper, however, because the amphipods are continuously growing (mean dry weight during the 4 to 6-week uptake experiments = 0.116 mg + 0.0143 mg/d), and the total body burden increases with time, even as the concentration decreases (Fig. 4). The constant k_r in Eq. 5 should not, therefore, be considered equivalent to an excretion rate.

Zinc uptake as a function of time

Zinc accumulation in *Hyalella* exposed to 4.6 μM Zn showed a rapid increase in the first 1 to 2 d, with no clear systematic variation for the next 6 weeks (Fig. 5). As for copper, an additional experiment was necessary to determine the initial rate of uptake of zinc. When fitted to Eq. 4, the data from the short-term uptake experiment gave an estimate for k of 3.5 d^{-1} (Table 1). Zinc concentrations in *Hyalella*, therefore, equilibrated faster than did copper concentrations. Using this estimate of k, Eq. 5 was fitted to the long-term uptake data giving k_r = 0.009 d^{-1} (Table 1). The estimate of k_r is not significantly different from 0 and the decrease in zinc from day 2 to 42 shown by the fitted line in Fig. 5 is not necessarily real. The mean zinc accumulated following exposure to 4.6 μM Zn (2.77 μmol/g; Table 2) is similar to that previously measured in *Hyalella* exposed to 4.9 μM (320 μg/L) Zn for 10 weeks (2.55 μmol/g (167 μg/g); Borgmann et al. 1993). Zinc is not, therefore, regulated in the same way as copper in long-term exposures.

Copper and zinc depuration

Copper that had previously been accumulated during a 1-week exposure was gradually excreted over a 2-week period with an apparent half-life of 3.5 days (Fig. 6). However, this apparent depuration rate includes growth dilution. To correct for growth (mean dry weight = 0.205 mg ± 0.0096 mg/d for the data in Fig. 6, based on linear regression of dry weight of sampled amphipods against time), the log-transformed copper concentration data were fitted to Eq. 6, giving an estimate for k_e of 0.16 d^{-1} (Table 1), and a true half-life of 4.3 d. This value of k_e is much smaller than the value of k obtained during the uptake experiments (Figs. 2 and 3; Eq. 4), indicating that the depuration curve is not a mirror image of the uptake curve.

Zinc that had previously been accumulated during a 1-week exposure was excreted more rapidly than copper. The log-transformed data were again fitted to Eq. 6, except that weight was replaced with (0.264 mg ± 0.0071 mg/d), the regression for weight against time for the zinc depuration experiment. This gave an estimate of k_e = 0.68 d^{-1} (Table 1), equivalent to a half-life of 1.0 d. The clearance rate for excess accumulated zinc was, therefore, over four times faster than the clearance of excess copper. However, as also observed for copper, the clearance rate (k_e) was significantly lower than the rate of equilibration during uptake (k), suggesting that zinc may also show saturation kinetics.

Metal uptake at varying water concentrations

The dramatic difference between k values (Eq. 4) obtained during both copper and zinc uptake, and k_e (Eq. 6) for

Table 1. Values (with 95% confidence limits given in parentheses) of the parameters fitted to Eqs. 3–6 by nonlinear least squares. Estimates for k_u are provided using two independent data sets, the concentration-series experiments (varying C_w) and the time-series experiments.

Parameter	Units	Equation and method of estimation	Copper		Zinc	
			Fixed constants	Estimate	Fixed constants	Estimate
k	d^{-1}	Eq. 4, 4-day time-series uptake	—	0.73 (0.55–0.91)	—	3.5 (2.6–4.4)
k_r	d^{-1}	Eq. 5, 6-week time-series uptake	$k = 0.73$	0.039 (0.024–0.053)	$k = 3.5$	0.009 (−0.001–0.020)
k_e	d^{-1}	Eq. 6, time-series depuration	—	0.16 (0.11–0.21)	—	0.68 (0.52–0.85)
C_{Xmax}	μmol/g	Eq. 3 (with $C_{X0} = 0$), 2-day uptake varying C_w	$k_e = 0.16$	3.60 (3.24–3.97)	$k_e = 0.68$	3.55 (2.98–4.11)
k_u	$L \cdot \mu mol^{-1} \cdot d^{-1}$	Eq. 3 (with $C_{X0} = 0$), 2-day uptake varying C_w	$k_e = 0.16$	0.55 (0.43–0.67)	$k_e = 0.68$	0.29 (0.21–0.37)
k_u	$L \cdot \mu mol^{-1} \cdot d^{-1}$	$k_u = (k - k_e)/C_w$, k and k_e from time-series experiments	$k = 0.73$ $k_e = 0.16$	0.76[a] (0.45–1.06)[b]	$k = 3.5$ $k_e = 0.68$	0.63[a] (0.39–0.87)[b]

[a]Using mean measured water concentrations of 0.755 μM Cu or 4.47 μM Zn in the time-series uptake experiments.
[b]Estimated from confidence limits for k and k_e listed above.

metal depuration (Table 1), indicates that $k_u C_w$ (Eq. 3) contributes significantly to the value of k. This means that there might be a finite number of copper and zinc binding sites that are partially saturated at the metal concentrations used, resulting in a hyperbolic relationship between C_{TB} and C_w at constant time (Eq. 3). To test this, we conducted 48-h uptake experiments over a range of metal concentrations from 0.05 to 16 μM (3.2–1000 μg/L). A short-term exposure was selected to ensure that survival would be adequate at the higher concentrations. Metal saturation did, in fact, appear to occur for both copper (Fig. 7) and zinc. C_{Xmax} and k_u were estimated simultaneously by fitting Eq. 3 to the data, with k_e set at 0.16 for copper and 0.68 for zinc, and C_{X0} set to 0 (Table 1). Copper saturation appears complete at about 4 μM (Fig. 7), and zinc saturation at about 8 μM.

The short-term uptake data for copper cannot be compared with long-term (10-week) uptake measured previously (Borgmann et al. 1993) because copper is gradually regulated and returns to control values (Fig. 2). However, the 48-h uptake compares favourably with the 1-week uptake measured previously (Fig. 7).

Estimation of uptake rates (k_u) from time- and concentration-series data

The value of k_u can be estimated independently from both the uptake curve (e.g., Fig. 3), using $k = k_u C_w + k_e$, and the concentration-series experiments (e.g., Fig. 7). Both procedures require estimates of k_e. The estimate of k_u obtained from the time-series uptake was 0.76 $L \cdot \mu mol^{-1} \cdot d^{-1}$ for copper. By comparison, the estimate of k_u from the concentration-series experiment was 0.55 $L \cdot \mu mol^{-1} \cdot d^{-1}$ (Table 1). Confidence intervals for estimates of k_u obtained from the two different approaches overlap. For zinc, on the other hand, the estimate of k_u was 0.63 based on the time-series uptake data, and 0.29 based on the concentration-series experiment (Table 1). The different estimates for k_u for zinc are reasonably close, considering the near overlap in confidence limits, but not identical.

Interaction of copper and zinc uptake

The effect of copper exposure on zinc uptake, and zinc exposure on copper uptake, was determined in some of the above experiments, supplemented with an additional 1-week uptake study. In the body size experiment (e.g., Fig. 1), amphipods were exposed either to single metals or to copper and zinc together. Exposure to both metals simultaneously resulted in a significantly ($P < 0.001$) increased copper uptake from 2.37 to 2.83 μmol/g, compared with amphipods exposed to added copper alone (Table 2). This was confirmed in the separate 1-week uptake experiment (an increase from 2.26 to 3.04 μmol/g; Table 2). On the other hand, exposure to both metals simultaneously resulted in a moderately significant ($P < 0.05$) decrease (4.28 to 3.76 μmol/g) in zinc uptake, relative to *Hyalella* exposed to added zinc alone (Table 2). However, this was not confirmed in the separate uptake study, where exposure to both metals resulted in zinc levels (3.10 μmol/g) not statistically distinguishable from amphipods exposed to added zinc alone (2.86 μmol/g; Table 2). Consequently, there is a clear stimulation of copper uptake by zinc, but no clear effect on zinc uptake by copper, during exposure to elevated concentrations of both metals.

Table 2. Geometric means (μmol/g dry wt.) of copper and zinc in *H. azteca*, number of amphipods analyzed (*N*), and standard deviation of log concentration (SD) for amphipods exposed to 0.79 μM (50 μg/L) Cu and (or) 4.6 μM (300 μg/L) Zn in several separate experiments.

Treatment	Copper			Zinc		
	Mean	*N*	SD	Mean	*N*	SD
Body-size experiment (e.g., Fig. 1)						
Unexposed	1.26*a*	63	0.073	1.19*a*	56	0.088
Cu exposed	2.37*b*	58	0.065	—	—	—
Zn exposed	—	—	—	4.28*b*	55	0.096
Cu + Zn exposed	2.83*d*	59	0.081	3.76*c*	59	0.146
Six-week uptake experiment (Figs. 2 and 5)						
Unexposed	1.13*a*	84	0.077	0.89*a*	79	0.130
Cu exposed	2.13*b*	62	0.117	0.93*a*	45	0.151
Zn exposed	1.18*a*	53	0.094	2.77*b*	69	0.162
One-week uptake experiment						
Unexposed	1.04*a*	7	0.053	1.05*a*	7	0.052
Cu exposed	2.26*b*	7	0.072	1.02*a*	6	0.048
Zn exposed	1.09*a*	8	0.039	2.86*b*	8	0.065
Cu + Zn exposed	3.04*c*	8	0.097	3.10*b*	8	0.100

Note: Means followed by different letters differ at ($P < 0.05$); means followed by *b* and *d* differ at $P < 0.001$.

In contrast to the above, exposure to either 0.79 μM Cu or to 4.6 μM Zn singly did not affect background concentrations of the other metal, as demonstrated in both the 6-week and separate 1-week uptake experiments (Table 2). The stimulatory effect of zinc on copper uptake was, therefore, only observed when both metal concentrations were elevated.

The relationship between metal uptake and toxicity

To relate short-term metal uptake to toxicity, the above results can be compared with previously determined chronic toxicity. An uptake period of 1 week was chosen for the following calculations as this ensures that maximum metal concentrations have been reached, but regulation of copper has not yet been achieved (Figs. 2–5). Copper and zinc concentrations accumulated in excess of background in 1 week ($C_{TB} - C_{Bk}$) were computed from the measured copper and zinc concentrations in the water using Eq. 3 and the estimates of $C_{X\max}$, k_u, and k_e listed in Table 1. The most sensitive indicator of chronic toxicity in *Hyalella* exposed to copper or zinc was mortality; growth and reproduction were not affected at concentrations below those reducing survival in 10-week exposures (Borgmann et al. 1993). Ten-week exposures represent a major portion of the life cycle of *Hyalella* at 25°C; reproductive maturity is reached in 4–5 weeks. Instantaneous mortality rates (m) were computed by regressing the logarithm of survival against time for each replicate using data summarized in Borgmann et al. (1993). Copper- and zinc-induced mortalities are relatively constant through time, giving linear log survival–time plots (Borgmann et al. 1993). The log transformed mortality data were then fitted to the relationship

$$[7] \quad m = m' + a(C_{TB} - C_{Bk})^n$$

where m' represents the control mortality rate and a and n are constants.

The mortality rate is relatively insensitive to small increases in C_{TB} above background, but increases rapidly when $C_{TB} - C_{Bk}$ exceeds about 1.5 μM for both copper and zinc (e.g., Fig. 8). The best fit to Eq. 7, for both copper and zinc, was obtained when n (rounded off to the nearest whole number) was set equal to 4. Estimates of m' were then 0.0552 week^{-1} for copper and 0.0539 week^{-1} for zinc. Estimates of a were 0.0055 (μmol/g)$^{-4}$week^{-1} for copper and 0.0145 (μmol/g)$^{-4}$week^{-1} for zinc.

Estimates of the body concentration resulting in statistically significant mortality were made as follows. ANOVA of log mortality rate against metal concentrations gave error mean squares of 0.045 47 for copper and 0.024 82 for zinc. Statistically significant ($P < 0.05$) differences for a pairwise comparison between the control and a metal exposure requires an F of 4.32 for copper (1, 21 df) and 4.28 for zinc (1, 23 df). The minimum significant difference between control and treatment ($(2F \times MS_{error}/4)^{0.5}$), assuming four replicate measurements each, becomes 0.313 for copper or 0.230 for zinc (log units), equivalent to a 2.1-fold increase in the copper and a 1.7-fold increase in the zinc experiment. The minimum significant mortality rate would, therefore, be 0.1135-week for copper and 0.0915-week for zinc. Rearranging Eq. 7, this would occur at a concentration of 1.8 μmol Cu/g, or 1.3 μmol Zn/g above background. Consequently, measurable chronic mortality would be predicted to occur if tissue concentrations exceed 1.8 μmol Cu/g, or 1.3 μmol Zn/g above background during 1-week exposures.

Fig. 5. Zinc accumulated by *H. azteca* exposed to 0 (△ and ▲) or 4.6 (□ and ■) μM Zn for 1–42 days. Symbols and lines are equivalent to those in Fig. 2.

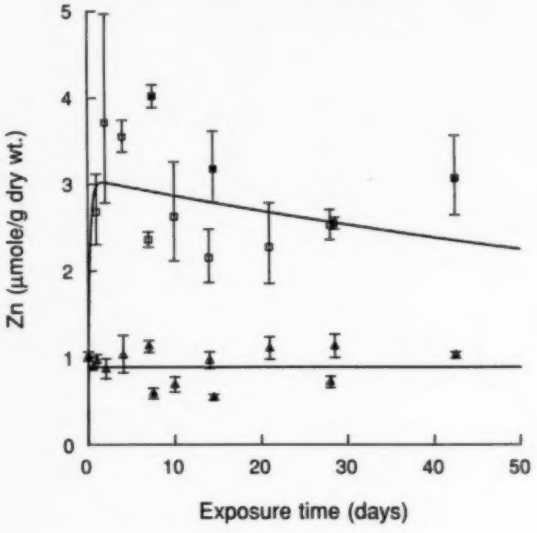
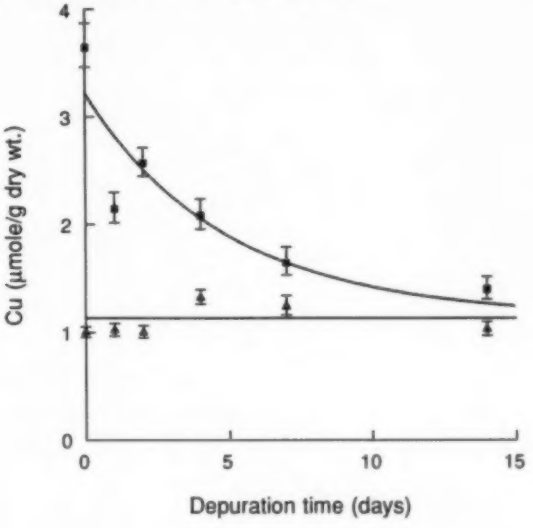


Fig. 6. Depuration rate of copper in *H. azteca* previously exposed to 0.79 μM Cu for 1 week (■) and comparison with control amphipods (△). Vertical bars indicate ±1 SE; each point is the mean of 15-31 measurements for previously exposed amphipods or 10 measurements for control amphipods. The overall mean copper concentration in control amphipods is shown as a horizontal line. The curve was fitted by least squares to log-transformed data using Eq. 6 in the text.



Discussion

Copper and zinc kinetics in *H. azteca* differ from observations made with other species in several ways. For example, the relatively small differences in body copper and zinc concentrations among *Hyalella* of different sizes (e.g., Fig. 1) contrasts sharply with the strong negative body size relationship previously shown for these metals in several species of *Gammarus* and other amphipods (Rainbow and Moore 1986; Johnson and Jones 1989). The difference is particularly striking because the most rapid increases in body copper and zinc with decreasing body size in these species were observed for animals below 2 mg dry weight (Rainbow and Moore 1986), yet the *Hyalella* analyzed were in the 0.02 to 2 mg dry weight range. The lack of a dramatic negative body size effect in *Hyalella* suggests that copper and zinc are not primarily adsorbed to the outside of the animals. This also suggests that the concentrations of these essential metals are controlled to some degree, and that background concentrations perhaps represent minimum tissue requirements.

Another surprising feature of *Hyalella* is its ability to regulate copper. The strong ability of several decapod crustaceans to regulate copper and zinc (e.g., White and Rainbow 1982; Bryan et al. 1986) is often not observed in lower crustacean forms. The data on amphipods is limited, but *Echinogammarus pirloti*, unlike shrimp, is unable to maintain constant internal copper and zinc concentrations with increasing metal concentrations in the medium (Rainbow and White 1989). The same applies to two species of talitrid amphipods (beachhoppers; Weeks and Rainbow 1991), although their semiterrestrial nature might make direct comparison with fully aquatic amphipods inappropriate. In contrast, *Gammarus duebeni* has been reported to maintain relatively constant internal zinc concentrations, but only up to 200 μg Zn/L (3.1 μM) in the medium (Johnson and Jones 1989). Xu and Pascoe (1994) suggested that *Gammarus pulex* might be able to regulate zinc because zinc concentrations in amphipods feeding on contaminated food were much lower than zinc concentrations in the food. However, uptake from water appears to follow a simple diffusion model (Xu and Pascoe 1993), as expected in animals not capable of regulation. Zinc appeared to be regulated in the amphipod *Allorchestes compressa*, but copper increased from 3.86 μmol/g (245 μg/g) to 8.12 μmol/g (8.12 μg/g) as water concentrations increased from 0 to 10 μg/L (0.16 μM) during 4-week exposures; further elevation in water concentration did not, however, increase copper uptake further (Ahsanullah and Williams 1991). *Hyalella* has been shown to maintain constant internal copper (but not zinc) concentrations after 10-week exposure, but not after only 1-week exposure (Borgmann et al. 1993). The data in Fig. 2 demonstrate that the return of internal copper concentrations to control levels is indeed a slow process in this species.

Another marked contrast between *Hyalella* and observations with other species is the difference between the uptake and depuration curves. In many studies, the half-life computed for the approach to equilibrium during uptake is equivalent to the half-life computed during depuration. This has, for example, been observed for zinc uptake and depuration in the amphipod *G. pulex* (Xu and Pascoe 1993), in crayfish (Giesy et al. 1980), and in mayflies (Hare et al. 1991). This is, however, clearly not the case for either

Fig. 7. Copper accumulated by *H. azteca* in 2 days as a function of measured copper concentration in the water. Vertical bars indicate ±1 SE; each point is the mean of 10 measurements (20 for control). The curve was fitted by least squares to log-transformed data using Eq. 3 in the text. Copper accumulated after 1 week exposure in previous experiments (*; Borgmann et al. 1993) is also shown for comparison.

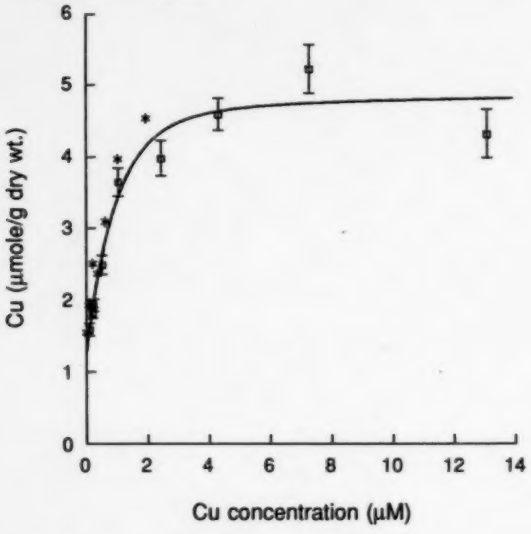
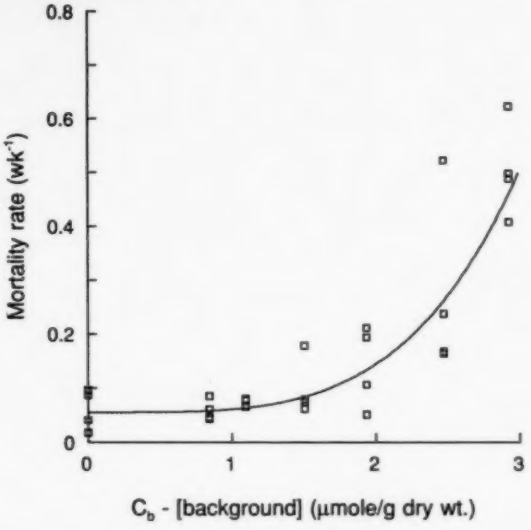


Fig. 8. Instantaneous mortality rate of *H. azteca* during chronic (10-week) exposure to copper (data from Borgmann et al. 1993) as a function of predicted copper accumulation above background in 1 week. The curve was fitted by least squares to log-transformed data using equation 7 in the text.



copper or zinc uptake and depuration in *Hyalella*. Copper and zinc excretion rates (k_e) are much slower than the approach to equilibrium during uptake (k; Table 1). Although several mechanisms may be responsible for this, the simplest explanation is that copper and zinc uptake by *Hyalella* is limited by the number of metal binding sites. The relative similarity in metal uptake rates (k_u) computed from the time-series and concentration-series experiments (Table 1) suggest such a model is reasonable.

A remarkable feature of the fitted curves for copper and zinc uptake, assuming that saturation occurs and that Eq. 3 correctly represents metal uptake in the short term (i.e., before regulation), is that the binding capacities (C_{Xmax}) for copper (3.60 µmol/g) and zinc (3.55 µmol/g; Table 1) are virtually identical. At first, this appears to suggest that both metals are bound to the same binding sites. However, this should result in competition for binding sites. This does not occur as neither metal interferes with the uptake of the other (Table 2). In fact, high zinc concentrations even enhance copper uptake slightly. The binding sites must, therefore, be different. Furthermore, a common binding site would suggest that, if metal regulation occurs, it should occur equally for both metals. Because copper, but not zinc, is regulated during long-term exposure, the binding sites for the metals probably differ.

In addition to C_{Xmax}, the uptake rates (k_u) of copper and zinc are also similar, to within a factor of about two (Table 1). The major difference between the kinetics of copper and zinc is the higher excretion rate (k_e) for zinc. This results in the faster depuration rate, faster equilibration during uptake, and saturation at higher water concentrations for zinc.

Zinc excretion rates in *Hyalella* (0.68 d^{-1}, half-life = 1 d) are similar to those observed in the amphipods *G. aequicauda* (52% loss in 1 day, Baudin 1982) and *Gammarus pulex* (0.35 d^{-1}, half-life = 2 d; Xu and Pascoe 1993). In contrast, no zinc excretion was observed in the amphipod *E. pirloti* (Rainbow and White 1989) or in talitrid amphipods (Weeks and Rainbow 1991). Zinc excretion rates in crayfish (0.029–0.080 d^{-1}, half-life = 9–24 d, Giesy et al. 1980) are slower than for *Hyalella*, as would be expected for this much larger crustacean, but still measurable. However, excretion rates for both copper and zinc are also slower in the isopod *Asellus aquaticus*; the half-life for zinc was 21–44 d and no excretion of copper was observed (van Hattum et al. 1993). It is interesting to note, however, that the relative excretion rates for copper and zinc (i.e., faster excretion of zinc) are the same as in *Hyalella*. On the other hand, copper and zinc excretion rates in the insect larva *Chironomus riparius* were very similar (0.12 and 0.11 d^{-1}, including growth dilution, half-life = 6 d; Timmermans et al. 1992a), with copper being similar to, and zinc much slower than in *Hyalella*. These excretion rates were, however, based on the uptake curve; excretion was not measured directly. Zinc excretion rates in caddisfly larvae (0.131 d^{-1}) and water mites (0.089 d^{-1}) were also similar (Timmermans et al. 1992*b*; also based on the uptake curve only). Zinc excretion in mayflies was slower (k_e = 0.022 d^{-1}; Hare et al. 1991).

Molting does not appear to be a major factor controlling copper and zinc kinetics in amphipods. Negligible quantities of copper or zinc were lost during molting in talitrid

amphipods, and whole body concentrations did not undergo significant changes during the molt cycle (Weeks et al. 1992). This may also apply to *Hyalella*. Adult *Hyalella* molt approximately once every 7 d at 25°C (U. Borgmann, unpublished data). Cast exuviae from *Hyalella* are very small and fragile and cannot be easily weighed. However, two pooled samples ($n = 6$ and 11) of exuviae shed between days 2 and 3 in the copper depuration experiment were analyzed and contained 0.0019 and 0.0024 nmol Cu/exuvia. This compares with a mean body concentration estimate of 0.42 nmol Cu/amphipod on day 0 and 0.27 nmol on day 3. Although this might be an underestimate of copper lost during molting, as all of the exuviae may not have been collected owing to their fragile nature, it appears that only very small amounts of copper are lost in the molting process. Molting is also not primarily responsible for the loss of zinc in *Hyalella*. The molting rate (approximately 0.14 d^{-1}) is much slower than the zinc depuration rate (0.68 d^{-1}) and cannot, therefore, account for a major portion of zinc depuration.

The above kinetic analyses are valuable for designing copper and zinc bioaccumulation experiments that can be useful in detecting metal pollution problems. The ability of *Hyalella* to regulate copper following long-term exposure (Fig. 2) precludes the use of copper measurements in wild animals as an indicator of elevated environmental availability of this metal. However, the very rapid equilibration during uptake (i.e., high values of k; Eq. 4) for both copper and zinc means that animals raised in the laboratory need to be exposed to environmental samples for only short periods (e.g., 4–7 d) after which an accurate assessment of available metal can be made. Furthermore, significant metal accumulation above background occurs at copper and zinc concentrations starting below those resulting in chronic toxicity (Borgmann et al. 1993). Background concentrations of copper ranged from 1 to 1.3 μmol/g dry weight, and for zinc from 0.9 to 1.2 μmol/g in the present experiments (e.g., Table 2). Toxicity does not occur until concentrations after 1 week of exposure are increased by a further 1.8 (copper) or 1.3 (zinc) μmol/g. Consequently, roughly a doubling (or more) in background copper or zinc is required to produce toxic effects, and this should be readily detectable. One-week-long metal uptake experiments with *Hyalella* are, therefore, potentially a powerful tool for rapidly assessing the impact of copper or zinc in contaminated water samples. Further research needs to be done to verify that the same body concentration – toxicity relationship holds when exposure is via contaminated sediments.

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EDTA toxicity and background concentrations of copper and zinc in *Hyalella azteca*

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Abstract: The effect of the strong metal-complexing agent EDTA on survival, growth, and copper and zinc concentrations in *Hyalella azteca* was studied to determine if metal concentrations in control amphipods represent minimum physiological levels. Exposure to EDTA concentrations of up to 560 μM for 1 week did not reduce metal levels (mean background = 1.2 μmol Cu/g and 1.0 μmol Zn/g dry weight), indicating that background metals are firmly bound to *Hyalella*. Chronic (4 week) exposure to EDTA resulted in increased mortality and reduced growth rates, both of which could be partially ameliorated by adding zinc to culture media. This implies that EDTA toxicity results, in part, from zinc deficiency. In spite of this, however, zinc concentrations in *Hyalella* were not reduced in amphipods raised in the presence of high EDTA, implying that the observed background zinc levels represent minimum physiological levels. Copper additions to media were ineffective at reducing EDTA toxicity. Nevertheless, control concentrations are probably close to minimum physiological levels for copper as well, because high EDTA did not appreciably reduce copper in *Hyalella* in chronic tests, in spite of its ability to impair copper uptake from water at moderate water concentrations.

Résumé : On a étudié l'effet du puissent chélatant EDTA sur la survie, la croissance et les concentrations de cuivre et de zinc de *Hyalella azteca*, afin de déterminer si les concentrations de métaux dans les amphipodes témoins constituent des niveaux physiologiques minimaux. L'exposition à des concentrations d'EDTA allant jusqu'à 560 μM pendant une semaine n'a pas abaissé les niveaux des métaux (concentrations naturelles moyennes : 1,2 μmole de Cu/g et 1,0 μmole de Zn/g, matières sèches), ce qui laisse croire que les métaux présents naturellement sont solidement liés à *Hyalella*. L'exposition chronique (quatre semaines) à l'EDTA a donné une hausse de mortalité et une diminution des taux de croissance, lesquels ont pu être améliorés en partie par l'ajout de zinc aux milieux de culture. Cela porte à croire que la toxicité de l'EDTA découle en partie de la déficience en zinc. Pourtant, les concentrations de zinc dans *Hyalella* n'ont pas été réduites chez les amphipodes élevés en présence de concentrations élevées d'EDTA, ce qui laisse supposer que les niveaux naturels de zinc représentent des niveaux physiologiques minimaux. Les ajouts de cuivre aux milieux de culture n'ont pas fait baisser la toxicité de l'EDTA. Quoi qu'il en soit, les concentrations de contrôle sont probablement proches des niveaux physiologiques minimaux pour le cuivre également, puisque des concentrations élevées d'EDTA n'ont pas réussi à faire baisser significativement les concentrations de cuivre dans *Hyalella*, lors de tests chroniques, malgré l'aptitude de l'EDTA à réduire l'absorption du cuivre de l'eau, à des concentrations moyennes.

[Traduit par la Rédaction]

Introduction

Metal concentrations in aquatic animals are potentially more useful indicators of environmental impacts than are concentrations in water. Metal toxicity is strongly affected by water chemistry and particulate matter, but bioaccumulation is affected by the same parameters. Consequently, toxicity should be more easily predicted from body concentrations than from water concentrations. This has been demonstrated, for example, for cadmium toxicity to *Hyalella azteca* (Borgmann et al. 1991). Several authors have recommended greater use of body burdens rather than water concentrations for evaluating potential contaminant toxicity, for both metals and organic contaminants (e.g., Connolly 1985; Landrum et al. 1992; McCarty and Mackay 1993). Furthermore, the frequently high degree of bioaccumulation often makes it easier to measure metal concentrations in animal tissues than in water.

Interpretation of the significance of body concentrations of two metals of common concern in freshwater

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environments, copper and zinc, presents special problems. These metals are essential to life and are readily accumulated and retained in animal tissues, even in pristine environments. In some species, such as decapods, these metals are often regulated, at least below some critical water concentration above which regulation breaks down. This is not the case with nonessential metals such as cadmium and lead (White and Rainbow 1982; Amiard et al. 1987). In more primitive invertebrates, including some amphipods, copper and zinc are not, or more poorly, regulated (Rainbow and White 1989; Borgmann and Norwood 1995). This makes lower invertebrates potentially more useful than regulators for assessing environmental contamination.

The significance of high background copper and zinc concentrations, however, must be clarified. Do these represent minimum essential background concentrations that are approximately constant? Alternatively, do the biochemical uptake mechanisms for these metals result in excessive accumulation in even moderately clean environments? Borgmann and Norwood (1995) suggested that metal concentrations in laboratory populations of *Hyalella* exposed to environmental samples for 1 week should provide a useful indicator of metal availability and potential chronic toxicity (metal levels in wild animals were not recommended as indicators of availability because *Hyalella* regulate copper in long-term exposures). However, copper and zinc are used extensively in laboratory buildings and equipment and their concentration in control animals could represent conditions already indicative of some degree of contamination, even if physiological effects are not detected. Copper and zinc concentrations in a number of wild amphipods were strongly size-dependent, suggesting that much of the metal may be adsorbed to the outside of the animal and that total body concentrations might only be indicative of physiological requirements in the largest animals (Rainbow and Moore 1986). However, Borgmann and Norwood (1995) observed no such body size effect for copper and zinc in laboratory-raised *Hyalella*, and metal concentrations were not corrected for body size. Metal kinetics in the presence of metal additions were analyzed as if the excess metal concentrations (i.e., metal concentrations above the average for controls in long-term uptake and depuration experiments, or metal concentration minus initial concentration in short-term (4-d) uptake experiments) acted independently of background metal concentrations (Borgmann and Norwood 1995). Pequegnat et al. (1969) attempted to estimate minimum zinc requirements of marine organisms from data on the fraction of known enzymes that require zinc for physiological activity, the number of zinc atoms required per molecule, and molecular weights. This approach was applied to both copper and zinc by White and Rainbow (1985), and recalculated for decapods by Rainbow (1992). Minimum copper and zinc concentrations ranged from 0.49 to 0.60 μmol Cu/g dry weight (31–38 μg/g) and from 0.14 to 0.31 μmol Zn/g (9–20 μg/g), but the actual observed concentration (e.g., 1.07–1.47 μmol Zn/g; 70–96 μg/g in shrimp) in animals that regulate copper and zinc might be a better estimate of actual metal requirements (Rainbow 1992).

In an attempt to clarify whether control copper and zinc concentrations in *Hyalella* represent true background (minimum physiological) requirements, we reduced metal availability to *Hyalella* by exposing them to the strong metal complexing agent EDTA (ethylenediaminetetraacetic acid). The toxicity of EDTA was determined and maximum EDTA concentrations were used in an attempt to "clean" the amphipods. The ability of EDTA to prevent accumulation of added copper and zinc was also tested. Finally, copper and zinc additions to culture media were given to amphipods exposed to toxic EDTA concentrations in chronic tests to test whether EDTA toxicity was, in fact, creating metal deficiencies in *Hyalella*.

Methods

Amphipods were cultured as described in Borgmann et al. (1989) and toxicity experiments followed procedures used previously (see Borgmann and Norwood (1995) for summary). The water used in all experiments originated from Lake Ontario and had a hardness of about 130 mg/L, alkalinity of 90 mg/L, and pH of 7.9–8.6. Experiments were initiated by placing 20 young or adult *Hyalella* in 250 mL of medium (water or water with EDTA and (or) copper and (or) zinc) in 500-mL Erlenmyer flasks with one, 5 × 10 cm piece of presoaked cotton gauze as substrate. Copper was added as $CuCl_2$, zinc as $Zn(NO_3)_2$, and EDTA as Na_2EDTA. Chronic (4-week) toxicity tests were conducted using 0- to 1-week-old *Hyalella*; media and food were replaced weekly. Short-term uptake experiments were conducted using 4- to 6-week-old adults exposed to media for 1 week; media were not renewed during the exposure period. Amphipods were fed 5 mg of Tetra-Min® fish food flakes per flask at the beginning of the experiment, and at weekly media changes during the chronic toxicity tests. An additional 5 mg of food was added once or twice per week whenever most of the previously added food was consumed. Additional feedings became more frequent as the animals matured and consumed more food. In all experiments, metals, EDTA, food, and gauze were allowed to equilibrate for approximately 4 h before amphipods were added. This was done because earlier (unpublished) experiments in which metal (Cu, Zn, Pb) concentrations were measured periodically in flasks with food and gauze after metal addition suggested that the most rapid changes in water concentrations due to precipitation or adsorption occurred within the first 4 h.

Copper and zinc concentrations in water and amphipods were measured using a Varian SpectraAA 400 graphite furnace atomic absorption spectrophotometer with Zeeman background correction. Copper was measured in a partition tube without modifier; zinc was analyzed using a platform and ammonium phosphate modifier. Background copper and zinc in the water ranged from 0.019 to 0.076 μM Cu (1.2–4.8 μg/L) and from 0.030 to 0.104 μM Zn (2.0–6.8 μg/L). Measured copper and zinc in water at nominal concentrations of 0.79 μM Cu and 4.6 μM Zn (Fig. 1) were 0.62 ± 0.12 μM Cu (mean ± SD; $N = 22$) and 5.34 ± 0.89 μM Zn ($N = 14$). Metals were not measured in water at nominal concentrations of 32, 100, and 320 μM Cu or Zn (Figs. 3 and 4). Metal analyses were performed on individual amphipods dried at 60°C, digested by addition of 25 μL of 70% nitric acid, and allowed to sit

Table 1. Control or initial concentrations of copper and zinc in several amphipod species, and theoretical minimum metal requirements and observed regulated concentrations in decapods (dry weight basis). For *Hyalella* in this study, the range in geometric means for individual experiments, overall geometric mean, standard deviation of log-transformed data, number of analyses, and the 95% confidence interval for the overall mean are shown.

Species	Copper		Zinc		Reference
	(μmol/g)	(μg/g)	(μmol/g)	(μg/g)	
Allorchestes compressa	3.86	245	2.13	139	Ahsanullah and Williams 1991
Corophium volutator	1.15	73	—	—	Eriksson and Weeks 1994
Echinogammarus pirloti	1.21–1.32	77–84	2.32	152	Rainbow and White 1989
Gammarus pulex	—	—	0.95–1.42	62–93	Xu and Pascoe 1993, 1994
Hyalella azteca	1.24–1.54	79–98	1.13	74	Borgmann et al. 1993
Range	0.90–1.58	57–100	0.74–1.20	48–78	This study; Borgmann and Norwood 1995
Geometric mean	1.17	74	1.00	65	
SD (*N*)	0.09 (483)		0.11 (455)		
95% CI	1.15–1.19		0.98–1.03		
Talitrid amphipods (two species)	1.05–2.20	67–140	2.65–2.83	173–185	Weeks and Rainbow 1991
Theoretical requirements in decapods	0.49–0.60	31–38	0.14–0.31	9–20	Rainbow 1992
Regulated concentrations in decapods	—	—	1.07–1.47	70–96	Rainbow 1992

for 1 week. Then 20 μL of 30% hydrogen peroxide was added, followed after 24 h by sufficient double-distilled water to bring the final volume to 0.5 or 1 mL before injection into the spectrophotometer.

Metal analysis of the Tetra-Min® fish food was performed both for the dry food and following extraction with experimental water containing various concentrations of EDTA. Several milligrams of ground fish food flakes were placed in centrifuge tubes, 10 mL of media was added, and the mixture was mixed on a vortexing mixer and allowed to sit for 4 h. The sample was then centrifuged, the media decanted, and the pellet remixed with distilled water and recentrifuged. The water was then decanted and the pellet dried in the centrifuge tube. The pellets were then digested and analyzed for copper and zinc using the same procedures used for the dried amphipods.

Statistical analysis of survival, growth, and body burden data (ANOVA) were performed using SYSTAT 5.0, with comparisons between treatments made by the Tukey method (comparison of all pairwise combinations) or Dunnett's test (comparison of all treatments with control). The term controls, as used throughout this paper, refers to animals not exposed to copper, zinc, or EDTA additions, but otherwise treated exactly the same as exposed animals and originating from the same batch of test animals as exposed animals in each separate experiment. Survival data were converted to mortality rates by regressing ln(survival) against time. Mortality rate, final wet weight, and body burden data were all logarithmically transformed before statistical analysis or least-squares fitting to equalize variances. For the chronic EDTA toxicity data, mortality rates (m) were fitted to the relationship

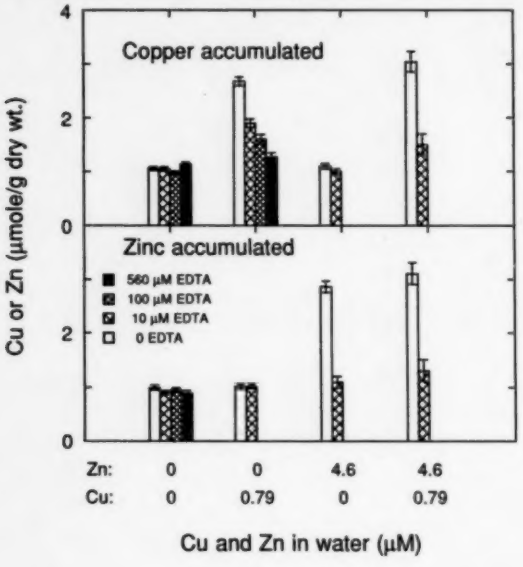
$$[1] \quad m = m' + a[\text{EDTA}]^n$$

where m' is control mortality and a and n are constants. Results of statistical analyses were back transformed to the original units for display in figures, resulting in slightly asymmetrical error bars.

Results

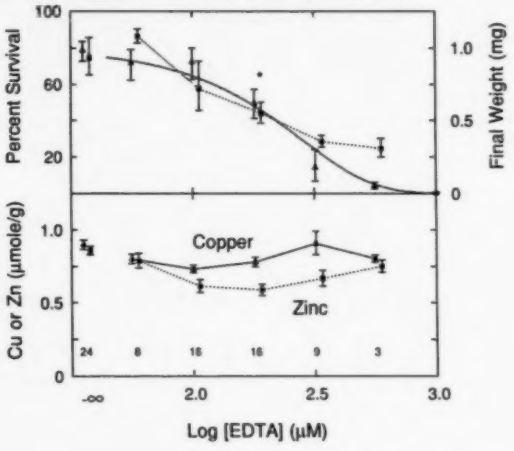
Mean copper and zinc concentrations in control *Hyalella* in separate experiments conducted between 1989 and 1994 ranged from 0.90 to 1.58 μmol Cu/g and from 0.74 to 1.20 μmol Zn/g dry weight, with an overall mean concentration of 1.17 μmol Cu/g and 1.00 μmol Zn/g (Table 1). Typical values for control metal concentrations and their errors are shown in Figs. 1, 2, and 4 (left side). In an attempt to ensure that a true uncontaminated control *Hyalella* body burden was obtained, a set of amphipods was treated identically to controls, but exposed to 1 μM (well above background Cu or Zn in water) EDTA during the long-term uptake study reported in Borgmann and Norwood (1995). The EDTA exposed amphipods contained slightly less copper (1.03 μmol/g) and zinc (0.86 μmol/g) than regular control amphipods in the same experiment (1.14 μmol Cu/g and 0.92 μmol Zn/g), but the differences were not statistically significant (Tukey test, ANOVA $P < 0.2$) and well within the normal range obtained on different dates (Table 1), suggesting that most of the copper and zinc measured in control *Hyalella* was not due to metal contamination. These data suggest that true background copper and zinc concentrations in *Hyalella* are close to 1 μmol/g dry weight, with slightly more copper than zinc usually present.

Fig. 1. Copper and zinc accumulated by adult *H. azteca* exposed to 0.79 μM (50 μg/L) Cu and (or) 4.6 μM (300 μg/L) Zn in the presence of 10–560 μM EDTA for 1 week. Error bars represent ±1SE (n = 6–20 for each bar). Zinc concentrations in all EDTA-exposed animals, and those exposed to copper alone, are not significantly different from controls. Copper concentrations in all copper-exposed amphipods are significantly higher than in controls, even at 560 μM EDTA, but are not significantly different from controls in animals exposed to zinc alone (Tukey test, $P < 0.05$). Metal concentrations for 100 and 560 μM EDTA-exposed animals were not measured where bars are missing.



A second check of the "cleanliness" of control *Hyalella* was made by exposing amphipods to a higher (10–560 μM) EDTA concentration for 1 week. Simultaneously, the ability of EDTA to prevent metal uptake by amphipods was estimated by exposing animals to both 10 μM EDTA and 0.79 μM (50 μg/L) Cu and (or) 4.6 μM (300 μg/L) Zn. These metal concentrations are approximately equally toxic in chronic tests and were used to study short- and long-term metal uptake in *Hyalella* (Borgmann and Norwood 1995). Exposure to 10 μM EDTA did not reduce copper or zinc in control animals, but it was successful at preventing any zinc uptake in the presence of 4.6 μM Zn (Fig. 1). Ten micromolar EDTA was capable of reducing, but not completely preventing copper uptake in the presence of 0.79 μM Cu (Fig. 1). Still higher concentrations of EDTA (100 or 560 μM) reduced copper uptake further, but again, could not prevent it completely (Tukey test, $P < 0.05$). Even such high concentrations of EDTA could not, however, reduce copper or zinc concentrations in control amphipods (Fig. 1). Depuration rates for excess copper and zinc (i.e., metal concentrations above background) are relatively rapid (Borgmann and Norwood 1995), so some decrease in control metal concentrations should have been observed if excess copper or zinc were present. This again suggests that our control amphipods are relatively clean and represent true background metal concentrations.

Fig. 2. Survival of (△, solid line, top panel), final wet weight of (□, dotted line, top panel), and copper (△, solid line, lower panel) and zinc (□, dotted line, lower panel) accumulated by 0- to 1-week-old *H. azteca* exposed to various concentrations of EDTA for 4 weeks. Error bars represent ±1SE (n = 4–6 in top panel; n is indicated by numbers under each point in lower panel). Survival and final weights at concentrations greater than or equal to values marked with an asterisk are significantly different from control (Dunnett's test, $P <$ 0.05). The curve drawn through the survival data was obtained by nonlinear regression of log mortality rate against EDTA concentration (Eq. 1 in text). Data for weight and zinc are offset slightly for clarity.



While the above experiments demonstrate that our control amphipods are relatively clean, in the sense that they do not contain appreciable amounts of readily extractable copper or zinc, they do not demonstrate whether *Hyalella* can survive with lower metal tissue concentrations. To test if this is so, it is necessary to raise amphipods under conditions of low metal availability. Young (0- to 1-week-old) *Hyalella* were, therefore, exposed to a logarithmic series of EDTA concentrations. Both survival and final body size were significantly reduced at EDTA concentrations of 180 μM and above (Fig. 2). Copper and zinc concentrations at the end of the exposure were significantly reduced at some EDTA concentrations, but the reductions were not large and not clearly related to the EDTA dose (Fig. 2). This suggests that the observed metal concentrations are close to those representing essential minimum requirements in this species.

Background metal concentrations in amphipods could be derived from either water or food. The above experiments (Fig. 1) demonstrate that high concentrations of EDTA can prevent zinc uptake and greatly reduce copper uptake from water. To estimate potential metal uptake from food, and the ability of EDTA at reducing this uptake, the amount of copper and zinc in the Tetra-Min® fish food before and after extraction with EDTA was measured.

Table 2. Geometric mean (μmol/g dry wt.), SD of log-transformed data, and number (*N*) of analyses of copper and zinc in both dry Tetra-Min® and Tetra-Min® extracted with 0–560 μM EDTA in tap water for 4 h.

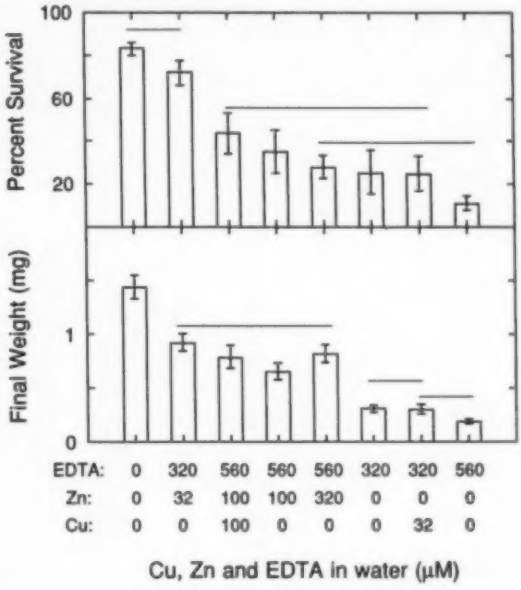
	Copper			Zinc		
[EDTA]	Mean	SD	*N*	Mean	SD	*N*
Dry	0.127	0.026	8	0.971	0.050	7
0	0.083	0.009	3	0.833	0.029	3
10	0.086	0.015	3	0.789	0.056	4
100	0.080	0.012	4	0.677	0.060	4
320	0.082	0.014	4	0.558	0.045	4
560	0.079		1	0.584	0.044	2

Some leaching of metals appeared to occur after extraction, even with water. However, this could be the result of loss of fine particulate matter on the sides of the centrifuge tubes or during decanting. When compared with the water only extraction, EDTA did not appear to remove any appreciable amount of copper from the Tetra-Min®, although the amount of zinc in the food decreased slightly (Table 2).

The actual amount of copper or zinc ingested in the food by amphipods is not known, but a rough comparison of the amount added to the experimental flask in food and the amount required for growth can be made. Assuming three additions of food per week at 5 mg each, and metal concentrations of 0.127 μmol Cu/g and 0.971 μmol Zn/g (Table 2), the total amount of metal added in food is about 2 nmol Cu and 15 nM Zn/week. Assuming a dry weight growth rate of about 0.06 mg/week (0.0071–0.0096 mg/d; Borgmann and Norwood 1995), a body concentration of about 1 μmol/g, and 10–20 animals/flask, the total metal requirement is about 0.6–1.2 nmol/week per flask. The total amount of zinc in the food, assuming it was all consumed, would, therefore, exceed the metal requirement of the animals. The copper added as food is very close to the amount actually required and might be limiting if all food is not consumed and copper is not accumulated from water.

To test whether the EDTA was causing toxicity by preventing assimilation of essential copper or zinc, whether from food or water, a 4-week exposure of young (0- to 1-week-old) amphipods was conducted at 0, 320, and 560 μM EDTA with varying concentrations of copper and (or) zinc additions to the medium. As in the previous experiment (Fig. 2), 320 and 560 μM EDTA caused significant mortality and decreased final body size. Addition of zinc at high, but below EDTA, concentrations (32–320 μM) resulted in significantly improved survival and growth when compared with equivalent EDTA concentrations without added zinc (Fig. 3). Addition of 32 μM Zn to 320 μM EDTA increased survival to a level not significantly different from the control, but growth was still lower than in controls. At 560 μM EDTA, zinc additions were less efficient at reducing EDTA-induced mortality, although some improvement did occur; growth was also substantially improved relative to 560 μM EDTA alone. Additions of copper did not improve survival or growth when added alone or in combination with zinc (Fig. 3). It appears,

Fig. 3. Survival and final wet weight of 0- to 1-week-old *H. azteca* exposed to various concentrations of copper, zinc and EDTA for 4 weeks, arranged in order of decreasing survival. Errors bars represent ±1SE (n = 8–12) and values not significantly different from one another (Tukey test, ANOVA, $P < 0.05$) are marked by a common overhead line.



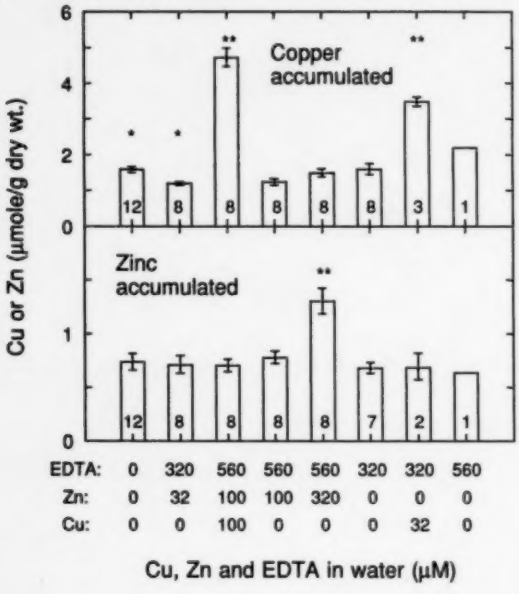
therefore, that EDTA toxicity is caused at least in part by a reduced availability of zinc. At high EDTA concentrations, an additional mechanism is also involved, but this does not appear to involve reduced availability of copper.

In the second chronic exposure experiment, as in the first (Fig. 2), EDTA alone did not have a marked effect on either copper or zinc concentrations in *Hyalella* (Fig. 4). Zinc concentrations in amphipods were elevated significantly at 320 μM Zn in the presence of 560 μM EDTA, and copper concentrations were elevated in both treatments containing added copper, in spite of the presence of EDTA. The metal concentrations added were, therefore, sufficient to reverse any metal limitation problems caused by the presence of EDTA, supporting the view that an additional mechanism of EDTA toxicity is also involved. Nevertheless, the inability of EDTA to reduce body burdens of copper and zinc below control levels, even at chronically toxic EDTA concentrations, and the ability of zinc to partially reverse EDTA toxicity, suggest that the measured concentrations in control amphipods are, in fact, close to the absolute minimum requirements for this species.

Discussion

Most of the copper and zinc measured in control *Hyalella* (means of 1.2 μmol Cu/g and 1.0 μmol Zn/g; Table 1) appear to represent minimum physiological requirements for this species. Exposure to high concentrations of EDTA, including those causing chronic toxicity, did not reduce

Fig. 4. Copper and zinc accumulated by 0- to 1-week-old *H. azteca* exposed to various concentrations of copper, zinc and EDTA for 4 weeks, arranged in order of decreasing survival. Error bars represent ±1SE (*n* indicated by numbers inside each bar).
**, Significantly different from all others (and each other in the case of copper; Tukey test, ANOVA, $P < 0.01$);
*, significantly different from one another ($P < 0.05$) but not from unmarked bars; unmarked bars are not significantly different from one another (Tukey test, ANOVA, $P < 0.05$).


Copper accumulated
Zinc accumulated
Cu or Zn (µmole/g dry wt.)
EDTA: 0 320 560 560 560 320 320 560
Zn: 0 32 100 100 320 0 0 0
Cu: 0 0 100 0 0 0 32 0
Cu, Zn and EDTA in water (µM)

copper or zinc concentrations measurably in most experiments (Figs. 1 and 4); some treatments did show a small statistically significant reduction, but this was not related to the EDTA dose (Fig. 2). This contrasts markedly with the relatively rapid loss of copper and zinc followed the transfer of *Hyalella* from copper- or zinc-spiked media to clean water (Borgmann and Norwood 1995), indicating that background copper and zinc are much more strongly bound to the amphipods than metals accumulated in excess of background from contaminated media. The ability of added zinc to partially reverse EDTA-induced toxicity in chronic exposures demonstrates that high EDTA concentrations cause zinc deficiency; survival and growth in 320 µM EDTA + 32 µM Zn were greater than in 320 µM EDTA alone, and in 560 µM EDTA + 100 µM Zn were greater than in 560 µM EDTA alone (Fig. 3). Nevertheless, zinc body burdens were not appreciably reduced, implying that measured zinc levels represent the minimum physiological requirement. It is notable that EDTA toxicity is expressed as a reduced growth rate, as well as increased mortality. This would be expected from an agent that caused nutrient deficiency (i.e., analogous to a decreased feeding rate). It was not possible to demonstrate copper deficiency at high EDTA concentrations; EDTA toxicity was not reversed by the addition of copper (Fig. 3). However, the relatively constant copper body burden with increasing EDTA exposure suggests that this metal is probably also at physiological minimum levels.

Hyalella probably accumulated most of their copper and zinc from food during exposure to EDTA. EDTA completely prevented zinc uptake from water, and greatly reduced copper uptake at 0.79 µM Cu (Fig. 1). Copper uptake from water in the absence of added copper (background Cu in water ≤0.076 µM) was probably negligible in the presence of ≥100 µM EDTA. Feeding rates were not determined, but rough calculations suggest that there is just sufficient copper in the food, and an excess of zinc, to meet growth requirements. Nevertheless, zinc appeared to be more limiting than copper during EDTA exposure. Perhaps EDTA is capable of extracting ingested zinc from *Hyalella* before it can be firmly bound in the animal. It is interesting to note that the excretion rate of excess zinc ($k_e = 0.68\ d^{-1}$) is substantially higher than that of excess copper ($k_e = 0.16\ d^{-1}$; Borgmann and Norwood 1995), suggesting that *Hyalella* has a greater ability to hold on to internal copper than internal zinc before it is firmly bound.

The term metal regulation, as it applies to *Hyalella*, requires some clarification. *Hyalella* is a relatively poor regulator, when compared with decapods (White and Rainbow 1982; Amiard et al. 1987), in the sense that it is incapable of maintaining constant body zinc at elevated zinc concentrations in the water and returns body copper concentrations back to control levels very slowly when copper in the water is elevated (Borgmann and Norwood 1995). However, it maintains fairly constant body concentrations of both copper and zinc in the presence of widely varying concentrations of free copper and zinc caused by the addition of high EDTA concentrations (Figs. 2 and 4). This suggests strong regulatory abilities for both metals. Clearly, *Hyalella* maintains constant body copper and zinc concentrations as long as waterborne metal availabilities are not high, probably while obtaining most of its metal requirements from food. Under normal (i.e., unpolluted) conditions, *Hyalella* is, physiologically speaking, an excellent regulator. The phrase "poor ability to regulate" these essential metals, therefore, applies only to *Hyalella*'s inability to prevent body concentrations from increasing in the presence of high water concentrations, and not to a general inability to keep copper and zinc at constant body concentrations.

The fact that EDTA was more efficient at preventing zinc uptake from water than copper uptake is surprising; 320 µM EDTA completely prevented uptake of zinc, but not copper, at 32 µM metal, and 560 µM EDTA completely prevented uptake of zinc, but not copper, at 100 µM metal (Fig. 4). Similarly, 10 µM EDTA completely prevented uptake of zinc at 4.6 µM, but did not prevent accumulation of copper, even at the lower copper concentration of 0.79 µM (Fig. 1). The binding constant for EDTA to copper is over 100-fold stronger than the binding of EDTA to zinc (Martell and Smith 1974). The free copper concentration must, therefore, have been much lower than the free zinc concentration at equivalent total metal and EDTA

concentrations. Apparently *Hyalella* has a much stronger ability to accumulate copper from water than zinc. The lower ability of EDTA to prevent copper uptake as compared with zinc uptake, the inability of EDTA to create a clear copper deficiency reversible by copper addition, and the much lower excretion rate of excess copper compared with zinc (Borgmann and Norwood 1995) all point to an extremely efficient mechanism for copper uptake and retention. Evolution of such a mechanism suggests that copper may be the more limiting of the two metals under natural conditions. This corresponds to an ability to regulate copper, but not zinc, during long-term chronic exposures (Borgmann and Norwood 1995), but it is not known if there is a connection between these observations.

A knowledge of correct background (control) metal concentrations is essential for an understanding of copper and zinc kinetics because background concentrations are subtracted from total metal concentrations when measuring excess metal accumulated from contaminated media (Borgmann and Norwood 1995). In our studies, these background concentrations appear to represent essential metal levels. Background copper concentrations in *Hyalella* ranged from 0.90 to 1.58 μmol/g and zinc from 0.74 to 1.20 μmol/g dry weight. In comparison, background (control and initial) concentrations of copper and zinc in other amphipods ranged from 1.05 to 3.86 μmol Cu/g and from 0.95 to 2.83 μmol Zn/g (Table 1). Background concentrations in *Hyalella*, therefore, overlap the lower range of these measurements.

Theoretical estimates of essential metal requirements, based on average copper and zinc concentrations in enzymes and haemocyanin, range from 0.49 to 0.60 μmol Cu/g dry weight and from 0.14 to 0.31 μmol Zn/g for several decapods (Rainbow 1992; Table 1). These values are lower than, but of the same order as those measured in wild crustaceans. The regulated concentrations of metals may, however, provide a more direct estimate of essential metal concentrations, at least in those species capable of maintaining a constant metal concentration over a range of concentrations in the medium. These range from about 1.07 to 1.47 μmol Zn/g in several decapods (Rainbow 1992) and are similar to background zinc concentrations in *Hyalella* (Table 1).

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